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CANCER RESEARCH

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A Critical Evaluation of the Current Status of Clinical Cancer Chemotherapy*

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The prognosis for the vast number of individuals in the world population who do not have cancer now but who will have overt manifestations of neoplastic disease in their lifetime may be dramatically improved by successful investigations leading to:

1. Knowledge of environmental factors which may produce malignant tumors and, then, prevention of the disease by appropriate control measures.

2. Specific diagnostic tests for incipient neoplastic growth.

3. Systemic chemotherapeutic agents for the selective destruction of malignant cells.

At the present time, the volume of research which is directly or indirectly applicable to these three primary objectives is so great and the optimism expressed in professional and lay publications is often so insistent that there is a widespread "feeling" that successful control of malignant disease is imminent. It certainly cannot be denied that important advances have been made in the understanding of the biology of malignant disease which may amply justify a sanguine outlook; at the same time, however, it may not be amiss to survey critically the practical applications of our knowledge to the problems of malignant disease in man in order to expose obvious lags between the laboratory and the clinic, to indicate the correlations and discrepancies between laboratory and clinical observations, and to illuminate gaps in our understanding which could profitably be studied further.

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The modern era of clinical cancer chemotherapy may reasonably be dated from the introduction of androgen control therapy of prostatic carcinoma 12 years ago. Since then, a number of drugs have been found to be useful in the management of a variety of human malignant diseases. It is the purpose of this review to evaluate the experience of the past 12 years with cancer chemotherapeutic agents and to present some problems which this experience has revealed.

In a provocative article written several years ago, Karnofsky suggested possible approaches to tumor chemotherapy (16), and in the present review chemotherapeutic agents will be categorized within this framework:

1. *Destruction of the "neoplastic component" of the malignant cell.*—Conceivably the "neoplastic component" might be a virus, a chemical compound, an abnormal gene, an aberrant enzyme system, or the absence of an enzyme system. The concept that there is such a component of neoplastic cells carries the implication that it is common to all malignant cells and therefore only one chemotherapeutic agent would be necessary for the control of all the varieties of neoplastic disease. At the present time the "neoplastic component" is a highly speculative entity and its definition must precede its destruction or modification.

2. *Damage to functional and chemical characteristics common to normal and neoplastic cells.*—This approach to the elimination of cancer by chemical compounds is exemplified by the cytotoxic drugs and will be discussed more fully presently.

3. *Stimulation of tissue defenses which limit the growth and spread of malignant cells.*—The evidence for inherent defense mechanisms against human cancer is tenuous and yet, on occasion, very per-

suasive. Thus, the rare but well documented cases of disseminated tumors which spontaneously regress (6) or the less infrequent instances of recurrent cancer many years after removal of the primary tumor suggest that there may be physiological mechanisms which can either destroy malignant cells or hold them in check for long periods. Certainly, intensive study is indicated of isolated patients in whom spontaneous tumor regression is observed; these natural experiments may provide the clues to define host resistance if such exists.

The venerable concept that the tissue defense against neoplastic cells resides in the reticuloendothelial system rests on extremely inconclusive evidence, and chemotherapeutic attempts to stimulate this system with antireticular cytotoxic serum or to augment the alleged anticancer function of it by the administration of splenic and other organ extracts have been uniformly worthless.

TABLE 1

CLASSIFICATION OF CANCER CHEMOTHERAPY ACCORDING TO MECHANISMS OF ACTION

Unfavorable alteration of environment of cancer cell	Modification of systemic intoxication produced by cancer	Damage to cellular functions common to normal and malignant cells of a tissue
Estrogens:	Androgens:	Urethan:
Prostatic cancer	Breast cancer	Leukemias
Breast cancer in males	Estrogens:	Myeloma
	Breast cancer	Folic acid antagonists:
	ACTH-cortisone:	Acute leukemia
	Lymphomas	ACTH-cortisone:
	Leukemias	Acute leukemia
		Nitrogen mustards:
		Lymphomas
		Leukemias
		Polycythemia vera
		Bronchogenic cancer

4. *Alteration of the environment of the cancer cell.*—This approach is exemplified by androgen control therapy of prostatic carcinoma; the rationale for this method of treatment as well as the results of such therapy will be presented later.

5. *Interference with the tumor blood supply.* It is recognized that malignant tumors characteristically stimulate the production of capillary endothelium thereby tending to insure an adequate blood supply for growth. Inhibition or destruction of the vascular circulation to tumors would theoretically be a useful chemotherapeutic approach. The closest approximation to this therapeutic method is the bacterial polysaccharide from *Serratia marcescens* which Shear (32), Creech (5), Seligman (31) and the clinical group at the Hahnemann Hospital (14) have studied carefully. Although the results in tumors of experimental animals are impressive, the toxicity of bacterial polysaccharide in man is forbidding. Strenuous efforts have been made to separate the systemic toxic actions and the tumor-necrotizing properties of the preparation, but, to date, this has not been possible. At the present time, therefore, this does not offer a clinically useful chemotherapeutic approach.

6. *Modification of systemic toxicity produced by cancer.*—The systemic toxic manifestations may determine the clinical picture of malignancy. Death in patients with malignant disease is not only caused by mechanical interference with the function of vital structures but also by subtle mechanisms which result in fever, anorexia, hypoproteinemia, intravascular hemolysis, depression of hematopoiesis, disturbances of carbohydrate metabolism, and probably many other metabolic abnormalities. These alterations lead to the clinical picture of toxemia and cachexia of malignancy. Although no specific correction of the disturbances just mentioned is recognized, nevertheless chemotherapeutic agents are available which pragmatically support the host against the ravages of his disease. Examples of these will be presented and their contribution evaluated.

Table 1 summarizes the foregoing paragraphs by grouping the currently employed clinical cancer chemotherapeutic agents according to their general mechanism of action. In the remaining portion of this review the chemotherapeutic agents will be presented in some detail, with an attempt to define their significance as practical therapeutic agents and as research tools.

UNFAVORABLE ALTERATION OF ENVIRONMENT OF CANCER CELL

The clinical application of this therapeutic principle is exemplified by androgen control therapy of disseminated prostatic carcinoma. At the present time castration, together with the administration of estrogens, is the accepted and generally employed regimen for the therapeutic management of the disease. This method of treatment rests on sound physiological grounds which may be summarized as follows:

a) Normal, adult prostatic epithelium is rich in an acid phosphatase (11).
b) Malignant prostatic epithelial cells are also rich in acid phosphatase, and when the tumor has extended beyond the confines of the prostate gland the serum acid phosphatase is elevated in about 65 per cent of cases (12).

On the basis of these facts, together with the known functional dependence of the prostate upon androgenic hormone, Huggins concluded that neoplastic prostatic cells retained at least some of the

characteristics of normal prostatic epithelium and therefore should undergo change when deprived of androgen. The hypothesis was put to test in man and proved correct (15).

Androgen control therapy results in subjective improvement, as manifested by loss of pain, increase of appetite, and a sense of well-being; evidences of objective improvement include decrease in size of soft tissue masses, healing of osseous metastases, spontaneous rise of hemoglobin, and degenerative changes in the tumor cells. In addition to the rapid subjective and objective improvement, androgen control therapy does alter the life expectancy of the patient with disseminated prostatic cancer. This can be demonstrated in survival statistics accumulated by Nesbit (22) from the experience of a number of American clinics. Thus, for example, the 3-year survival after the institution of effective androgen control therapy by orchiectomy and estrogen administration is between 50 and 60 per cent, whereas only about 20 per cent of untreated cases survive for this period.

In spite of this encouraging picture, it must be recognized that death due to prostatic cancer is inevitable unless an intercurrent pathological process interrupts the course of the primary disease. Why should this be so? The chief and obvious explanation is that, in spite of widespread destruction of the cancer, some viable malignant cells can always be demonstrated in biopsies of the primary tumor or metastases. But why are not these survivors held in check? The answer to this question is not ready. Two possibilities merit consideration: First, the remaining cells may represent mutants which differ from the parent population in not being androgen-dependent; or, second, the elaboration of androgenic steroids by the adrenal cortex may be sufficient to permit the survival of some of the cancer cells. On the basis of the latter hypothesis, Huggins in 1943 adrenalectomized patients who had become refractory to androgen control therapy. Because of the difficulty of maintaining life with the then available substitution therapy, the experiment was inconclusive. With the advent of cortisone, Huggins has re-explored this possibility, and his preliminary reports indicated that a dramatic remission could be induced by adrenalectomy in patients refractory to conventional therapy. The precise mechanism by which this change is produced is still to be explained, although Huggins still favors the idea that adrenalectomy merely removes another source of androgenic hormone. In any event, this heroic procedure is not the solution to the problem of prostatic cancer refractory to androgen control therapy; already there have been patients who have relapsed after initial benefit, and a number fail to show any significant improvement.

It is to be remembered that the intimate cellular mechanisms of prostatic epithelium which depend upon androgenic hormone are still unknown. It is most probable that androgen modifies the function of an enzyme or enzymes. When more accurate knowledge is available on this basic issue, it is likely that more effective chemotherapy of prostatic cancer will follow.

One of the most interesting biological paradoxes in human neoplastic disease is highlighted by the pragmatic observation that carcinoma of the male breast is effectively controlled by orchiectomy plus estrogen administration. This relatively rare tumor is not usually observed by physicians until after it has reached the stage of local inoperability. It is therefore fortunate indeed that androgen control therapy is usually followed by prolonged remissions measurable in years.

Modification of Systemic Intoxication Produced by Cancer

A. Hormones in carcinoma of the female breast

1. *Androgens.*—The basis for hormone therapy of breast cancer was laid by Leo Loeb (21), who demonstrated that castration of female mice with a high incidence of spontaneous mammary carcinoma significantly reduced the frequency of occurrence of the tumor. This indication of the importance of estrogenic hormone on the genesis of breast cancer was fortified by observations of Lacassagne and his associates, who showed in a variety of experiments that the female sex hormone stimulated the formation of spontaneous tumors (18). Lacassagne first suggested the use of male sex hormone for the therapeutic management of breast cancer.

At this point, the experimental studies failed to provide supportive evidence for androgenic therapy of mammary carcinoma. It was found that testosterone was effective in decreasing the incidence and frequency of the tumor in mice only if therapy was started shortly after birth and continued throughout the life of the animals. Under these conditions, ovarian function was effectively suppressed. If testosterone was administered shortly before maturity of the mice, the frequency of spontaneous tumors was unaltered, and if therapy was instituted after the appearance of the cancer no significant modification of the course of the disease was observed.

In spite of these disappointing results, testosterone was given to women with advanced breast cancer. Initially, with low doses, no beneficial effects were noted; the clinical observations of

Adair and Hermann using large doses of testosterone (300 mg/week) showed that improvement could be effected in a proportion of the cases (1).

Recently, a subcommittee of the Council on Pharmacy and Therapy chaired by Nathanson has reported its analysis of 450 cases of advanced mammary carcinoma treated with androgens (4). Eighty per cent of the patients experienced subjective improvement. This was most notable in patients with symptomatic osseous metastases. These women had relief of bone pain, increased sense of well-being with improved appetite, and weight gain. Only 20 per cent, however, showed objective evidences of improvement, although liberal criteria were used. Rarely were there morphological changes in soft tissue metastases, and rarely was there roentgen evidence of healing of osteolytic lesions. The median survival time from the onset of treatment of the group which improved on hormone therapy was 1 year; those that failed to have clinical benefit had a median survival of 8 months. This is not a significant difference.

Before discussing the mechanism of action of androgens on human breast cancer, the effect of estrogens on the same tumor will be described.

2. *Estrogens.*—Estrogenic therapy of inoperable breast cancer was proposed on a highly speculative theory of hormonal imbalances for which no convincing supportive evidence has been produced. In spite of this, pragmatically, estrogens do provide benefit in properly selected patients. The most important basis for the use of estrogens, after it has been determined that conventional surgical or radiotherapeutic measures are inappropriate, is the age of the patient and her temporal relationship to the menopause. Estrogens should be limited to patients at least 5 years postmenopausal. Disregard of this condition may be followed by fulminating progression of the malignant disease.

In a series of 300 cases on estrogen therapy, 40 per cent showed improvement (4). For the majority of these, the benefit was largely subjective; however, in some there was unequivocal regression of local skin disease and in pulmonary and lymph node metastases. As in the series of androgen therapy, the median survival time of those who failed to show improvement was 8 months; those that did respond had a median survival interval from the onset of hormone therapy of 15 months. This is a statistically significant prolongation of life. It should be noted that, in general, the prognosis of carcinoma of the breast is more favorable in the older age group, because of the less virulent biological characteristics of malignancy.

The mechanism of action of androgens and estrogens in human breast cancer has not been established. In the reviewer's opinion, the evidence for a fundamental action of the hormones on the neoplastic breast cells is minimal. Morphological changes, even when there has been evident regression, are rare, and the frequency of objective tumor regression is far less than that of subjective improvement. Not infrequently there may be regressive alterations in one metastatic area while other metastases show progression. Since effects of the gonadal hormones on protein metabolism, on osteoid deposition, on sodium and calcium metabolism, and on proliferation of squamous epithelium are well recognized and documented (26), it is possible and probable that the clinical changes produced are in the nature of nonspecific support.

An apodictic statement on the mechanism of action of the sex hormones in breast cancer is unwarranted, however, because of the occasional case in which gross and microscopic changes indistinguishable from those due to radiation are demonstrable. The isolated instances may represent spontaneous remissions but also may be due to a direct deleterious effect of the hormones on cancer cells.

B. HYPERCORTICISM IN THE TREATMENT OF LYMPHOMAS AND LEUKEMIAS

With the advent of adrenocorticotropic hormone (ACTH) and cortisone, which modify the signs and symptoms of so many diseases, it was natural that they should be tried empirically in cancer patients. Sufficient experience has now been acquired in the latter category to assign the indications and limitations of these drugs with some assurance.

Cortisone and ACTH can modify the manifestations of the malignant lymphomas (lymphosarcoma and Hodgkin's disease) but do not affect the neoplastic cells significantly. Their action is apparent only while they are being administered and within days after discontinuation the clinical evidences of disease return. The chief value of ACTH and cortisone in the treatment of the lymphomas is sharply limited to a particular phase of the disease. It is common in the final stages of patients with Hodgkin's disease or lymphosarcoma for a pancytopenia to develop, in part due to the effect of the disease on hematopoiesis and in part due to treatment with x-ray and/or drugs which depress bone marrow function. At this time the patient usually has severe constitutional symptoms such as fever, malaise, and anorexia. In the past, since further definitive therapy was contraindicated by the bone marrow depression, this was recognized as the preterminal phase of the pa-

tient's illness. To a certain extent ACTH and cortisone have altered the prognosis.

Following the induction of hypercorticism, the temperature may return to normal, a sense of well-being may be restored with improved appetite and strength. During this state the anemia may be easily corrected by transfusions, and there may be a spontaneous return of bone marrow function. If the latter event occurs, then once again definitive treatment can be reinstituted and a significant remission produced. If hematopoiesis fails to return adequately, the manifestations of the underlying disease inevitably reappear in spite of continued therapy with ACTH or cortisone, and death soon occurs.

There is no effective therapy for acute leukemia of adults, and the chief therapeutic weapon has been blood transfusions. Thrombocytopenia with purpura is an almost unvarying characteristic of the natural history of this dyscrasia, and death is not infrequently due to bleeding. It has been found that in a significant proportion of patients the purpuric manifestations can be controlled temporarily with ACTH or cortisone. As in the case of the lymphomas, only symptoms are modified rather than the fundamental disorder; there is no alteration of the leukemic process in the bone marrow, and, if the patient does not have a spontaneous remission, death due to the disease occurs.

Thus, it can be seen that ACTH and cortisone are temporizing drugs which improve signs and symptoms of constitutional toxicity but do not modify the tumor. The precise mechanism of action of the hormones is unknown. It is possible that the anti-inflammatory effects noted in infections, rheumatoid arthritis and other diseases accounts for the observed changes in the symptoms of these malignant tumors.

Damage to Cellular Functions Common to Normal and Malignant Cells

A. URETHAN

Ethyl carbamate was selected by Haddow and his associates for trial in human neoplastic disease because of its inhibitory effect on the growth of the Walker carcinoma 256 (13). This chemical compound depresses normal bone marrow function and has been found to lower the peripheral leukocyte count of patients with chronic myelocytic and chronic lymphocytic leukemia. It is one of several drugs which can accomplish this end, and, since the results are neither quantitatively nor qualitatively superior to splenic irradiation, it is used only when radiotherapy is contra-indicated or not available.

Although urethan has been tried as a therapeutic agent for a variety of human tumors, its only other application is in the treatment of multiple myeloma. Rundles and his co-workers have studied the effects of urethan particularly in myeloma, and in their experience about one out of three patients shows subjective and objective evidences of improvement (29). This is manifested by relief of bone pain, decrease in the frequency of myeloma cells in the bone marrow, and a drop in the serum globulin; rarely, there is evidence of bone repair of the osteolytic skeletal lesions. The experience of other clinical investigators has been less fortunate than that of Rundles, but this may be in part due to selection of cases and duration of therapy. It has been found by Rundles that the earlier treatment is instituted in the course of the disease, the greater the likelihood of inducing a remission; he has also emphasized that it is necessary to continue urethan administration for at least 6 weeks in spite of absent clinical response before it is permissible to conclude that the drug will not benefit the patient.

The major toxic reactions to urethan aside from gastrointestinal upsets are due to depression of normal hematopoietic function. This re-emphasizes the fact that the chemical compound interferes with cellular mechanisms which are common to normal as well as to leukemic and myeloma cells.

Although urethan has long been known to physiologists, pharmacologists, and botanists, their studies have failed to clarify its mechanism of action precisely (27). There is cytological evidence of interference with cell division, and therefore, circumstantially, the site of action is probably on nucleoprotein or nucleic acids. Investigations by modern methods to determine whether this is a primary or a secondary mechanism have been inconclusive.

B. FOLIC ACID ANALOGS (AMINOPTERIN, A-METHOPTERIN, ETC.)

The antifolic acid drugs were introduced by Farber (8) to the clinic as a consequence of the laboratory investigations of Lewisohn which directed attention to the importance of folic acid in abnormal growth (20). The folic acid analogs are extremely potent chemical compounds which have found their chief application in the treatment of acute leukemia in childhood. Tremendously exciting changes may be produced in a moribund child by this group of drugs. The hemogram returns toward normal with a decrease in the total leukocyte count and the percentage of blast forms; the erythrocyte and platelet counts rise spontaneously with an associated cessation of purpuric

manifestations; visceral leukemic infiltrations and lymphadenopathy disappear; the hectic temperature returns to normal, and subjectively the child improves dramatically with recovery of appetite, interest, and joy in his surroundings. When it is recalled that prior to 1948 no therapeutic regimen could modify the course of acute leukemia it is readily understood why the folic acid analogs have been accorded so much attention.

If the response just described occurred regularly and if the remissions produced were protracted, then the folic acid antagonists could justifiably be considered of major therapeutic importance. Unfortunately, it is necessary to record many qualifications to the optimal effects noted (7). Only about one third of the cases of acute leukemia in children have a complete clinical and hematological remission. Another group comprising about 20 per cent of the total show only a partial hematologic response but are nevertheless improved symptomatically. The remaining 50 per cent of cases may suffer toxicity from the drugs, and hemorrhagic phenomena appear which, along with the depredations of the primary disease, contribute to their death early in the course of treatment. When a remission is induced, its duration is measurable in months, and retreatment with the folic analogs is progressively less effective with eventual complete refractoriness of the leukemia to the drugs. The brilliant and fascinating studies of Burchenal (3) and Law (19) and their associates have demonstrated that the development of resistance to aminopterin or A-methopterin in mouse leukemia is due to selection of refractory mutant cells by the agents. There is every reason to believe that the same mechanism pertains to the development of resistance in human leukemia. There are several additional important features about folic acid antagonist treatment of the acute leukemias. These drugs are notably ineffective in the management of this blood dyscrasia in adults. So striking is the age dichotomy that it is reasonable to suspect that the biology of the disease in children and adults is fundamentally different.

The ultimate effect of definitive chemotherapy of acute leukemia on survival cannot be stated conclusively at the present time, but the evidence indicates that the median duration of life in children who do show a remission is about 12 months. This is approximately twice the expected survival in untreated cases or in those that fail to have a remission from treatment (7).

Finally, the toxic manifestations produced by these drugs are impressive. In addition to the bone marrow depression which may result in pancytopenia with all of the complications associated with this condition, administration of aminopterin and A-methopterin may also cause hemorrhagic necrosis of the epithelium of the alimentary tract clinically recognizable by ulcerative stomatitis, gastrointestinal symptoms, or bloody diarrhea. Careful observation of the patient with discontinuation of medication when signs or symptoms of intoxication appear is necessary in order to avoid disastrous complications.

At the beginning of this section mention was made of the fact that the folic acid antagonists were the first drugs ever to modify the course of acute leukemia in children. In spite of this, the potential hazards of toxicity, the expense of repeated hospitalizations, the severe psychological trauma to the parents as they witness remissions and exacerbations of the disease in their child, and the disappointing ultimate outcome even when therapy is initially successful must be considered by the physician before initiating therapy with these drugs.

In the past few years there has been intensive study of the role of folic acid in cellular metabolism and the correlated problem of the mechanism of action of the folic acid analogs. The demonstration by Sauberlich (30) and others that folic acid is converted *in vivo* to the biologically active citrovorum factor or folinic acid has helped to clarify the previously unanswered question of the inability of folic acid to reverse the toxicity of the antifolic drugs. Nichol and Welch (23) have shown that aminopterin blocks the conversion of folic acid to folinic acid, and certainly this is an important mechanism of biological action of the drugs. In addition to this, however, the folic acid analogs act as competitive inhibitors of the citrovorum factor, thereby interfering with those enzyme functions in which the vitamin is associated as a coenzyme. Although all the enzyme systems in which folinic acid play a role have probably not been defined, it is known that the vitamin is concerned with the transfer of an active one carbon fragment in a variety of reactions. Of particular interest in this latter category is the evidence which demonstrates that the incorporation of the carbon atom in position two of the purine nucleus is dependent upon an enzyme system which includes folinic acid (36). It may therefore be concluded that a basic part of the mechanism of action of the antifolic drugs is to interfere with the synthesis of nucleic acids.

C. CORTISONE AND ACTH

In acute leukemia of children, these hormones also produce subjective and objective improvement. In about 70 per cent of untreated cases

clinical improvement is demonstrable. One-half of these have hematological remissions as well as clinical remissions. The duration of the remissions is variable, lasting from 2 weeks to 9 months. As with the folic acid analogs, however, refractoriness of the disease inevitably develops, so that the over-all prolongation of life is disappointingly small. A fascinating fact of practical importance is that refractoriness to adrenal steroid therapy does not necessarily imply refractoriness to the folic acid analogs, so that it is possible to induce remissions in the disease with the latter drugs when the former have become ineffective; the converse is also true.

At the present time cortisone is usually employed initially in the treatment of childhood acute leukemia. This appears to be the agent of choice, because the toxic manifestations of hypercorticism are less dangerous than the toxicity of the antifolic drugs. Theoretically, combination therapy of acute leukemia by the simultaneous administration of cortisone and A-methopterin seems sound, since presumably the drugs are acting through different mechanisms. In practice, however, the efficacy of this regimen is no greater than either drug alone.

There is little precise information on the mechanism of action of cortisone in acute leukemia. Biochemical studies on surviving tissue slices have revealed depression of several enzyme systems (34, 35), but the relevance of these observations to the antileukemic action of the hormone is not clear. Possibly of greater pertinence are the studies on the effect of cortisone on nucleic acid metabolism of transplanted mouse tumors which indicate an increased cellular concentration of nucleic acids when the growth of the tumors is inhibited.[1]

D. NITROGEN MUSTARDS

The nitrogen mustards have been intensively and extensively investigated for their clinical value in the therapy of neoplastic disease. The discussion of the clinical application of nitrogen mustards will consider primarily the use of the commercially available drug, methylbis(2-chloroethyl)amine hydrochloride (HN2) which is now sold under the trade name of Mustargen. For the safe and proper use of this toxic drug it is imperative to know the clinical pharmacology as well as its indications and contra-indications.

The notable actions of therapeutic doses of HN2 on the normal organism are destruction of lymphoid tissue and depression of bone marrow function. In the application of the drug to the treatment of certain neoplasms, it is impossible to avoid the effects on these normal tissues so that toxicity is an invariable accompaniment to therapeutic action. With recognition of this major limitation to effective therapy, nitrogen mustard nevertheless contributes to the practical management of the malignant lymphomas, certain blood dyscrasias, and bronchogenic carcinoma.

The results of HN2 therapy in over 400 cases of Hodgkin's disease have been reported in the literature, and there is impressive agreement among clinical observers that the chief value of the drug is in the management of this disease. This does not imply that chemotherapy has supplanted conventional radiotherapy, but rather that the two methods of treatment supplement and complement one another.

A primary criterion for consideration of nitrogen mustard in Hodgkin's disease is evidence of dissemination of the neoplasm. It is appreciated that Hodgkin's disease may be multicentric in its origin rather than metastasizing from a primary focus, but this is an academic issue. From a practical standpoint experience has amply demonstrated that aggressive radiotherapy of clinically localized disease may be followed by protracted remissions measurable in years. Since it is now known that HN2 cannot cure Hodgkin's disease or any other neoplastic process, it is not justifiable to employ a systemic agent for the control of a localized area of disease.

When, on the other hand, Hodgkin's disease is obviously disseminated as evidenced by generalized lymphadenopathy, abdominal visceral infiltration, intrathoracic disease, osseous involvement, or fever and other constitutional symptoms, then a choice between radiotherapy and chemotherapy can be considered. Nitrogen mustard is also valuable in the initial treatment of Hodgkin's disease compressing vital structures in the mediastinum or the spinal cord as well as for the symptomatic relief of pruritus and bone pain associated with generalized disease.

One of the most constant effects of nitrogen mustard in Hodgkin's disease is the reduction of fever to normal temperature within a few days. Associated with this reponse there is rapid subjective improvement with greatly improved appetite and dramatic increase in sense of well-being. Although radiotherapy also produces this type of subjective and objective improvement, the time interval from onset of treatment to benefit is considerably longer than following nitrogen mustard therapy.

In considering the immediate prognosis of radiotherapy or nitrogen mustard therapy of Hodgkin's disease, the natural course of the illness

[1] J. Gang and E. Hirschberg, in preparation.

and the number and types of treatments previously employed are important factors influencing the therapeutic response. In the advanced stages of the disease the benefits of HN2 therapy may be brief, incomplete, or absent. Before the terminal phase of the disease has been reached, however, an appreciable beneficial response can be expected in about 60 per cent of the courses administered. The duration of the remissions induced by nitrogen mustard is variable; however, a median duration of 6 weeks is probably a fair and reasonable characterization of the remission interval.

Since the consensus of all clinical observers is that nitrogen mustard makes its chief contribution in the treatment of Hodgkin's disease, it is of interest and significance to assess quantitatively the results of therapy in this disease. In our clinic an analysis of the first 5 years of experience with nitrogen mustard revealed that there was no significant prolongation of life in patients treated with HN2 and radiotherapy in alternating courses, as compared to that of patients who had been treated by radiotherapy alone (9). However, even though nitrogen mustard fails to alter the duration of the disease, evidence was accumulated which showed that the amount of x-ray therapy required was materially reduced by the use of chemotherapy and that patients were maintained in an asymptomatic state for a greater proportion of the disease course; the economic burden of the illness was also significantly decreased, owing to shorter hospitalizations and fewer x-ray treatments.

Clinical results follow nitrogen mustard therapy of disseminated lymphosarcoma which are qualitatively similar to those described in Hodgkin's disease. The responses are less uniform, however, and the histological type of the tumor is an important factor determining the response. In the blood dyscrasias, nitrogen mustard can successfully reduce the peripheral blood count of patients with chronic leukemia but shows no advantage over radiotherapy or other drugs such as urethan or arsenic. The chemical compound is of no value in the management of acute leukemia of children or adults.

Nitrogen mustard has been given therapeutic trial in a wide variety of inoperable carcinomas. Instances of temporary regression of a number of epithelial tumors have been recorded, but only bronchogenic carcinomas have responded with sufficient regularity to merit consideration of the chemical compound for routine treatment when surgery is impossible. Subjective and/or objective improvement follows the administration of nitrogen mustard in approximately 75 per cent of inoperable cases of bronchogenic carcinoma. The remissions so induced are usually brief (2 weeks to 2 months), and subsequent courses of therapy are less effective.

There is still much to be learned about the mechanism of action of nitrogen mustard. A number of facts are known, however, which allow a general interpretation of the fundamental biological activity of this class of chemical compounds.

When nitrogen mustard is placed in solution at the pH of body fluids, it undergoes a chemical transformation to a cyclic immonium cation. The significance of this intramolecular rearrangement lies in the fact that the transformation product is highly reactive chemically. It is now well established that the biological activity of HN2 is due to the avidity with which its cyclized form interreacts with chemical radicals of biological importance.

Much effort has been devoted to the identification of the particular cellular elements which are attacked by the nitrogen mustards. At the present time the accumulated evidence from biochemical, histochemical, and morphological studies points to the nucleic acids as the cellular site of action of these chemical compounds. Cytological experiments have demonstrated mitotic inhibition with specific alteration of chromosomal structure in cells exposed to extremely low concentrations of mustard. Since the chromosome is largely composed of complex nucleoprotein, the morphological evidence suggests that the mustards react with these chemical entities. This hypothesis is strengthened by the finding that nitrogen mustards can induce heritable mutations in the fruit fly and certain fungi and can inactivate viruses, particularly those rich in desoxyribonucleic acid. Nitrogen mustards decrease the viscosity of polymerized nucleic acids, and there is evidence from isotope tracer studies that DNA synthesis is depressed in animals given toxic doses of the drug. Circumstantial as the evidence is, its weight strongly supports the concept that nitrogen mustard disturbs the synthesis and/or utilization of nucleic acids (25). As was intimated in the section on the folic acid antagonists, interference with the function of these important chemical compounds is incompatible with cell survival.

E. TRIETHYLENE MELAMINE (TEM, 2,4,6-TRIETHYLENE-IMINO-S-TRIAZINE)

After the initial dramatic remissions induced by HN2 in clinical cancer chemotherapy, hundreds of chemical congeners were synthesized and tested in the laboratory. A few have been given clinical trial, but no advantages over HN2 have been

noted. Recently, a heterocyclic compound with 3 ethyleneimine radicals, triethylene melamine, has been studied in both the laboratory and the clinic. Although this compound is not yet generally available, the preliminary results indicate that it will merit inclusion in the cancer chemotherapeutic armamentarium.

Qualitatively, TEM closely duplicates HN2 in its therapeutic and toxic activity. There are two advantages of the drug over HN2: (*a*) it is active when given by mouth and (*b*) the frequency and severity of nausea and vomiting is appreciably less than after nitrogen mustard. These two factors facilitate the ambulatory therapy of patients with malignant lymphomas. Preliminary clinical reports have been made by Karnofsky (17), Wright (37, 38), and others (2, 10, 24, 28, 33).

DISCUSSION

The preceding sections have summarized briefly the facts about drugs which are generally employed today in the treatment of human malignant disease. There are at least two obvious major limitations of current cancer chemotherapy. With the exception of the hormones, the chemical compounds which are used have significant toxicity to normal as well as neoplastic cells. This precludes curative therapy, since the treatment necessary for destruction of the tumor would be lethal to the host also. Another limitation is the small number of tumor types which respond to drugs. Urethan, the folic acid antagonists, cortisone, and the nitrogen mustards play a role in the treatment of the malignant lymphomas and leukemias. Statistically these constitute a small fraction of all the malignant neoplastic diseases. In spite of the contributions made by the sex hormones in the management of prostatic and breast cancer, there still is a tremendous need for chemical compounds which will be useful in the treatment of epithelial tumors.

At the present time there are two general philosophies which may be applied to the selection of cancer chemotherapeutic agents: the rational and the empirical. No one would deny that the development of antitumor drugs on a rational basis is intellectually more appealing than the trial and error method. On the other hand, it must be recognized that our understanding of normal and neoplastic cell physiology is still so incomplete that it is unlikely that practical applications to therapy can be anticipated by this method for a long time. Pressed by the urgent need for effective treatment of disseminated neoplastic disease, there has been an expansion of the empirical approach to the problem. In large part this has consisted of screening chemical compounds for their gross effect on the growth of transplantable tumors in mice, rats, and rabbits. From such screening programs urethan and TEM have emerged. The folic acid antagonists, nitrogen mustard, and cortisone were all tried empirically in man and only subsequently was their antitumor action confirmed in experimental tumors. The sex hormones which modify human epithelial neoplasms fail to have a demonstrable effect on animal tumors.

Over 10,000 compounds have been screened against animal tumors. It is perhaps justifiable to question the methodology of selecting drugs for human malignant disease, since from this appreciable experience only four classes of useful chemical compounds have emerged. There are several issues that merit consideration. It must be recognized that with the possible exception of mouse leukemia there is no correlative evidence to demonstrate that transplantable or spontaneous tumors in laboratory animals are biologically directly comparable to human tumors. One apparent discrepancy between animal and human tumors is in their relative growth rates. The cells of experimental tumors divide rapidly, and at any instant the majority of them are in an active phase of division. In the screening experiments the experimental drug is perforce administered for a short time period, and the end points are size, weight, or morphology in the case of solid tumors and survival time in the case of leukemias. Human epithelial tumors are relatively slow-growing, and the morphological evidence indicates that only a small fraction of the cells are in active mitosis at one time. Since it is likely that cancer chemotherapeutic agents, like antibacterial drugs, can only destroy cells which are susceptible by virtue of active metabolism and division, it follows that effective drugs against human epithelial tumors will have to be administered chronically and be well tolerated for long time periods by the normal host cells.

It may be significant that the drugs which are active in the currently employed animal tumor screening tests are effective principally against the most rapidly growing human tumors, the lymphomas and leukemias. Is it possible that the screening technics are missing potentially useful compounds against other tumors? The workers in the field of experimental cancer chemotherapy screening are the first to recognize this possibility. What could be done about it? It is suggested for the consideration of those interested in the problem that more subtle end points than the ones now in use might indicate chemical compounds which fail to produce dramatic changes in tumor growth after brief administration but which might modify

the growth of tumors when given over long periods of time.

Earlier, the question was raised concerning the relevance of studies with animal tumors to human neoplastic disease. It is not unreasonable to conclude that until such time as it is clearly demonstrated that a particular animal tumor is comparable to a specific human tumor, the screening of potential chemotherapeutic agents should be conducted in humans with malignant disease. Immediately, moral and emotional objections come to mind, but these need not concern us at the present time because the technical difficulty of evaluating chemotherapy in human cancer is too great to make human screening feasible. It is true that the nitrogen mustards, folic acid analogs, and cortisone were found to be active first in human lymphomas and leukemias. However, it also must be recognized that in these diseases there are many objective changes which facilitate the evaluation of a drug, such as rapid fluctuation in lymph node masses, fever, and peripheral blood counts and morphology. In the statistically important group of human neoplastic diseases, the epithelial cancers, the chronicity of tumor course, together with the relative unreliability of clinical evaluations based on changes of x-ray shadows, alteration in size of soft tissue masses, alleviation of pain or improvement in other subjective symptoms, constitute a sufficient deterrent to effective screening. Again, it is possible that the application of available knowledge of cell physiology to this problem would lead to the development of methods to assess objectively and quickly the effect of chemical compounds on human epithelial tumors. If this does occur, then serious consideration should be given to chemotherapeutic screening in man.

It would be unwarranted to close a review of current clinical cancer chemotherapy which has emphasized the limitations of the available drugs without stating their value as well. It is undeniable that the chemical compounds discussed have contributed significantly to the comfort of patients with disseminated malignant disease, permitting periods of happy and useful living not previously possible. This, coupled with the fact that their application has stimulated productive laboratory and clinical research on neoplastic disease, provides a reasonable basis for their acclaim.

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The Effect of Exogenous Gonadotrophins on the Development of Experimental Ovarian Tumors in Rats*

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Since our first description of the induction of an ovarian tumor by transplantation of an ovary into the spleen of castrated rats (4), numerous reports have confirmed this result (7–11, 14).

The types of cellular change that occur in the transplanted gonad vary with the experimental animal employed. The ovarian tumor may metastasize in mice but not in rats (9); luteal proliferation without luteoma occurs in the guinea pig (12), but the monkey is refractory to this type of tumor induction (16); and different rat strains may show great resistance to testicular tumor formation, whereas ovarian tumors are readily obtained (6).[1]

In spite of this somewhat variable response, it has been suggested that the development of the tumor is the result of inactivation by the liver of the hormones elaborated by the transplanted ovary (5). This would permit uninterrupted excessive stimulation by the pituitary and the release of the ovary from estrogenic inhibition. The possibility of elaboration of growth-promoting substances by the transplanted ovary must also be considered. Silverberg *et al.* (15), by subcutaneous transplantation of the hypophysis in mice with intrasplenic ovarian grafts, accelerated tumorigenesis. Miller *et al.* (13) demonstrated increased gonadotrophin in castrated mice with intrasplenic ovarian grafts by the use of parabionts. Bernstorf (1), however, recently reported that the intrasplenic ovary was refractory to the administration of exogenous gonadotrophins in the mouse.

The experiments to be described show that, in the Long-Evans strain of rats, the administration of exogenous gonadotrophin accelerates the development of both luteoma and granulosa-cell tumors when an ovary is transplanted to the spleen of a castrated rat.

MATERIALS AND METHODS

The rats employed in this study were of the Long-Evans strain, and they were divided into groups as follows:

Forty-eight young, adult, female animals were given intramuscular injections of 5 units of luteotrophin,[2] 3 times weekly. These were divided into Group A, which consisted of 29 castrated rats in which an ovary was transplanted to the spleen, and Group B, which consisted of nineteen rats in which one ovary was transplanted to the spleen and the other left *in situ*.

Thirty-nine young, adult, female animals were given intraperitoneal injections of 5 units of pregnant mares' serum (PMS),[3] 3 times weekly. These were divided into Group C, which consisted of 29 castrated rats in which one ovary was transplanted to the spleen, and Group D, which consisted of ten rats in which one ovary was transplanted to the spleen and the other left *in situ*.

Group E consisted of 25 young, adult, female rats which were castrated, had one ovary transplanted to the spleen, and received no exogenous hormones.

The rats were maintained on a complete stock diet and water available ad libitum. The animals were sacrificed at intervals of from 14 to 388 days, and a complete autopsy was performed. Histologic sections of the endocrine glands, genital organs, heart, lungs, liver, kidney, and spleen of each animal were examined.

RESULTS

The animals of Group A (Table 1), which received luteotrophin, were sacrificed at intervals of from 14 to 230 days. Eleven of these animals had

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[2] Luteotrophin supplied as Luteotrophin Squibb by Squibb & Sons, New York.

[3] Pregnant mares' serum supplied as Gonadin by the Cutter Laboratory, Berkeley, Calif.

adhesions which permitted blood from the spleen to bypass the portal circulation, and the transplants failed to show growth or neoplastic development. Of the remaining eighteen animals, four showed luteomas which were found at 160, 180, 202, and 219 days, respectively. However, in animal No. 3604 (Table 1), a granulosa-cell tumor was found 175 days after transplantation (Figs. 3, 4).

In Groups B and D, in which one ovary was transplanted to the spleen and the other left *in situ*, the intrasplenic ovary failed to undergo growth, despite the administration of exogenous gonadotrophin (Figs. 1, 2). There was hypertrophy of the ovary *in situ*, but it was no greater than that reported in a previous series of untreated animals (2, 4).

Of the 29 animals of Group C, which received PMS (Table 2), there were fourteen animals with splenic adhesions that permitted circumvention of the portal circulation, and characteristic growth or neoplastic development of the transplant was not observed in these animals. In the fifteen animals without adhesions, two luteomas were seen at an interval earlier than in any control group, namely, at 90 and 127 days. Three of the granulosa-cell tumors occurred as early as 126, 150, and 194 days, which is half or less of the usual time of previously reported series (3).

The histologic pattern of the luteoma and of the granulosa-cell tumors of this investigation was identical with that described in previous reports (3, 5). No striking cytologic alteration in the pituitary was discerned that could be attributed to the exhibited exogenous gonadotrophins. The staining technic employed was hematoxylin-eosin and Poirrier's blue-eosin. Differential cell counts were not made.

The animals of Group E (Table 3), which received no gonadotrophin, served as a control group and reaffirmed the time relationships for the appearance of the luteoma that were described in the original reports (3, 5). These animals were sacrificed at fairly regular intervals during a 270-day

TABLE 1

EFFECT OF LUTEOTROPHIN* ON INTRASPLENIC OVARY IN CASTRATED FEMALE RATS (GROUP A)

RAT NO.	TRANSPLANT Age (days)	TRANSPLANT Size† (mm.)	ADHESIONS‡	UTERUS OR VAGINA§	PATHOLOGICAL FINDINGS#
3592	14	2	0	e.s.	several c.l.; fol.
3593	14	2	0	e.s.	several c.l.; fol.
3588	30	5×6	0	a	large c.l., developing fol.
3591	30	3×5	0	e.s.	large c.l., developing fol.
3589	56	7×6	0	a	large c.l., developing fol.
3590	56	5×6	0	a	large c.l., developing fol.
3606	68	8×8×20	0	a	large poorly defined c.l.
3607	68	5×6	0	a	cystic transplant with c.l.
3605	83	8×14×15	0	a	cyst lined by c.l. and fol.
3529	112	6×10	1+	e.s.	cyst lined by c.l.
3528	112	6×6	1+	a	c.l.
3527	140	10×10	0	a	occasional c.l., small cyst, rare fol.
3530	140	3×4	4+	e.s.	few small c.l.
3983	149	5	4+	e.s.	poorly defined c.l.
3991	149	5×6	0	e.s.	cyst, c.l., fol.
3986	160	6	0	a	luteoma, c.l. around periphery
3987	160	5×4	1+	a	involuting c.l.
4226	171	scar	4+	e.s.	scar with atrophic c.l.
3526	167	3	4+	e.s.	scar with atrophic c.l.
3523	167	1	4+	e.s.	scar with atrophic c.l.
3522	167	2	4+	e.s.	scar with atrophic c.l.
3604	175	16×18×20	0	e.s.	cystic granulosa-cell tumor
3982	180	5×5×6	0	a	luteoma
3988	190	4×5	0	a	distinct small c.l.
3743	202	5×7	0	e.s.	luteoma
3990	202	3×5	1+	a	small atrophic c.l.
3989	219	8×10×18	1+	e.s.	cyst, poorly defined c.l.
3985‖	219	3×5	1+	e.s.	luteoma
3984	230	3×4	0	a	cyst lined by c.l.

* Luteotrophin administered 5 units 3 times weekly, intramuscularly.

† A single figure denotes the diameter of a spheroidal mass in the spleen.

‡ The adhesions that circumvented the portal circulation are graded 1 to 4.

§ a = atrophic; e.s. = estrogenic stimulation.

c.l. = corpora lutea; fol. = follicles.

‖ In animal 3985 the adhesions did not bypass the portal circulation.

period in order to re-establish the earliest time for the development of the tumor. This was found to be 210 days for the luteoma. No granulosa-cell tumors were seen in the 270-day experimental period. In a previous larger group in which the animals were examined at shorter intervals, the usual time for the appearance of the luteoma was 6–7 months; however, in one animal it was found as early as 157 days (3). Fourteen animals with adhesions from the spleen to the parietal abdominal wall that circumvented the portal circulation showed a few partially involuted corpora lutea, but no evidence of luteal proliferation or neoplastic change in the ovarian transplant.

DISCUSSION

The observations of this and other laboratories indicate that a characteristic series of changes occurs after the transplantation of an ovary into the spleen in the castrated rat. The transplant first shows necrosis; regeneration is then evidenced by the development of showers of primordial follicles which undergo luteinization. The outstanding feature is the continuous appearance of primordial follicles and the failure of involution of the corpora lutea. The process continues for about 6 or 7 months and seems to change abruptly when there is an overgrowth of luteal tissue, which rather quickly pushes aside the remaining corpora lutea and takes on a uniform cellular appearance that fits the commonly described pattern of a luteoma. This tumor generally increases in size and may sometimes be cystic. After another interval, which usually is 10–12 months following transplantation, there appear in the luteoma clusters of cells of the granulosa type. These are arranged in the varying patterns ordinarily noted in the human granulosa-cell tumor. At present there is no explanation for

TABLE 2

EFFECT OF PMS* ON INTRASPLENIC OVARY IN CASTRATED FEMALE RATS (GROUP C)

Rat no.	Age (days)	Transplant Size† (mm.)	Adhesions‡	Uterus or vagina§	Pathological findings#
3468	54	6	0	a	many large c.l.
3213	59	5	0	a	large c.l., some cystic
3469	80	6	0	a	many c.l., no fol.
3457	90	18×11×12	0	a	luteoma, large c.l. around periphery
3219	93	4	1+	e.s.	cystic fol., distinct c.l.
3218	93	9×7	0	a	many c.l., small fol., some cystic
3451	115	4	1+	e.s.	few c.l., occasional developing fol.
3453	120	4	4+	e.s.	few c.l., occasional developing fol.
3211‖	126	20×10×8	0	a	early granulosa-cell tumor
3214	127	15×10×10	0	a	large luteoma
3452	150	1	4+	no tissue	scar
3462	150	5	4+	no tissue	many c.l.
3446	150	15×10×10	0	a	granulosa-cell tumor
3217	157	1	4+	e.s.	scar
3212	158	5	4+	e.s.	atrophic luteal cells with no c.l.
3466	175	5	4+	e.s.	small c.l., rare fol.
3220	193	4	4+	e.s.	cystic developing fol., small c.l.
3216	194	10	0	a	granulosa-cell tumor
3444	194	4	4+	e.s.	small cluster c.l.
3456	242	3	4+	e.s.	few cystic c.l.
3454	242	10	0	a	luteoma
3447	242	9×6	4+	e.s.	cyst lined by c.l.
3465	287	3	0	a	luteoma
3449	287	7×3	0	a	luteoma
3458	287	20	0	a	luteoma
3459	287	3	4+	e.s.	dense atrophic c.l.
3463	287	4	1+	e.s.	solid c.l.
3445	388	27×17×13	0	e.s.	granulosa-cell tumor
3448	388	50	0	a	granulosa-cell tumor

* PMS administered 5 units 3 times weekly, intraperitoneally.

† A single figure denotes the diameter of a spheroidal mass in the spleen.

‡ The adhesions that circumvented the portal circulation are graded 1 to 4.

§ a = atrophic; e.s. = estrogenic stimulation.

c.l. = corpora lutea; fol. = follicles.

‖ Luteoma with early granulosa-cell tumor in a cystic transplant that retained corpora lutea (See Figs. 5, 6).

the appearance of the luteoma before the granulosa-cell tumor. If adhesions which develop following transplantation permit blood from the spleen to bypass the liver, the above sequence of events does not occur.

Exceptions to this usual pattern of development of the tumor seem to occur under stimulation by exogenous hormones. This is illustrated in Figures 5 and 6, taken from animal No. 3211, that had been treated with PMS. There is a small area of luteoma separate from the intact, well defined corpora lutea. The transplant also has a cystic zone lined by compressed corpora lutea. Small nests of granulosa-cell elements are scattered through the luteoma. This is in contrast to the findings in control animals where corpora lutea are no longer recognizable by the time the granulosa-cell tumor appears.

In the present work it is apparent that PMS accelerated the development of the ovarian tumors, as evidenced by a shortened interval for the appearance of both the luteoma and granulosa-cell tumor in the intrasplenic ovary of castrated Long-Evans female rats. Luteotrophin did not stimulate luteoma production, but in one rat a granulosa-cell tumor was found as early as 175 days (Figs. 3, 4). The relative lack of effect by luteotrophin may be owing to either the commercial preparation per se or the route of administration. However, the possibility of an antihormone effect must also be considered, as it will be noted that in two animals of the PMS group, namely, No. 3465 and No. 3449, and in all four animals of the luteotrophin group, namely, Nos. 3986, 3743, 3982, 3985, the luteomas were 3–7 mm. in diameter, which is considerably smaller than that usually found in the controls.

The acceleration of tumorigenesis by PMS lends credence to the hypothesis that increased or uninhibited pituitary stimulation of the intrasplenic gonad is of prime importance in the genesis of these experimental tumors. However, pituitary stimulation may not be the only factor involved in the formation of these tumors, because the intrasplenic ovary of the animals of Groups B and D, where one ovary was left *in situ*, did not show any stimulation by exogenous gonadotrophin. Current similar investigations on hypophysectomized animals may elucidate this problem.

TABLE 3

TRANSPLANTATION OF OVARY TO SPLEEN OF CASTRATED FEMALE RATS (GROUP E: CONTROLS)

Rat no.	Age (days)	Transplant Size* (mm.)	Adhesions†	Uterus or vagina‡	Pathological findings§
1820	30	4×5	0	a	c.l., developing fol.
1817	60	5×5	0	a	c.l., developing fol.
1812	90	6×7	0	a	c.l., developing fol.
1815	90	4	4+	e.s.	atrophy of c.l.
1810	90	3	4+	e.s.	atrophy of c.l.
1813	120	5	1+	a	atrophy of c.l.
1811	120	6×7	0	a	large c.l., developing fol.
1805	150	5×6	0	a	large c.l., developing fol.
1807	150	6×6	0	a	large c.l., developing fol.
1580	150	2	4+	e.s.	luteal cyst
1756	150	1	4+	e.s.	atrophy of c.l.
1808	150	3	4+	e.s.	atrophy of c.l.
1803	180	10×8×5	0	a	luteal cyst
1732	185	1	4+	e.s.	fibrous scar
1809	190	2	4+	e.s.	fibrous scar
1579	210	8×8×10	0	e.s.	luteoma
1731	210	1	4+	e.s.	atrophy of c.l.
1802	210	4	4+	e.s.	cyst with fibrous scar
1744	240	12×12×10	0	e.s.	luteoma with c.l. present
1730	240	6×10×6	0	a	luteoma
1738	240	3	4+	e.s.	scar
1742	270	10×8×8	0	e.s.	luteoma
1729	270	1	4+	e.s.	atrophy of c.l.
1728	270	4	4+	e.s.	occasional c.l.
1727	270	3	4+	e.s.	occasional c.l.

* A single figure denotes the diameter of a spheroidal mass in the spleen.
† The adhesions that circumvented the portal circulation are graded 1 to 4.
‡ a = atrophic; e.s. = estrogenic stimulation.
§ c.l. = corpora lutea; fol. = follicles

SUMMARY

In castrated female rats with one ovary in the spleen that received 5 units of luteotrophin intramuscularly, 3 times weekly, a luteoma appeared at 160 days, and one granulosa-cell tumor was found

in 175 days. In similarly prepared animals injected intraperitoneally with 5 units of pregnant mares' serum, 3 times weekly, luteomas appeared as early as at 90 and 127 days, and granulosa-cell tumors at 126, 150, and 194 days. Unilaterally castrated female rats with one ovary in the spleen served as controls to each of the above groups, and the exogenous hormones in both cases did not stimulate the intrasplenic ovary.

These findings are evidence that exogenous gonadotrophins may accelerate the development of both luteomas and granulosa-cell tumors in the rat ovary transplanted to the spleen of castrates. This lends credence to the hypothesis that increased or uninhibited pituitary stimulation of the intrasplenic ovary may be an important factor in the development of such tumors in the rat.

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Fig. 1.—The atrophied ovarian tissue in the spleen shows a single corpus luteum composed of a few luteal cells surrounded by fibrous tissue. Above it are distorted follicles, two with deeply stained follicular cells (×50). Animal No. 3196 with right ovary *in situ*, left ovary in spleen, received PMS for 196 days.

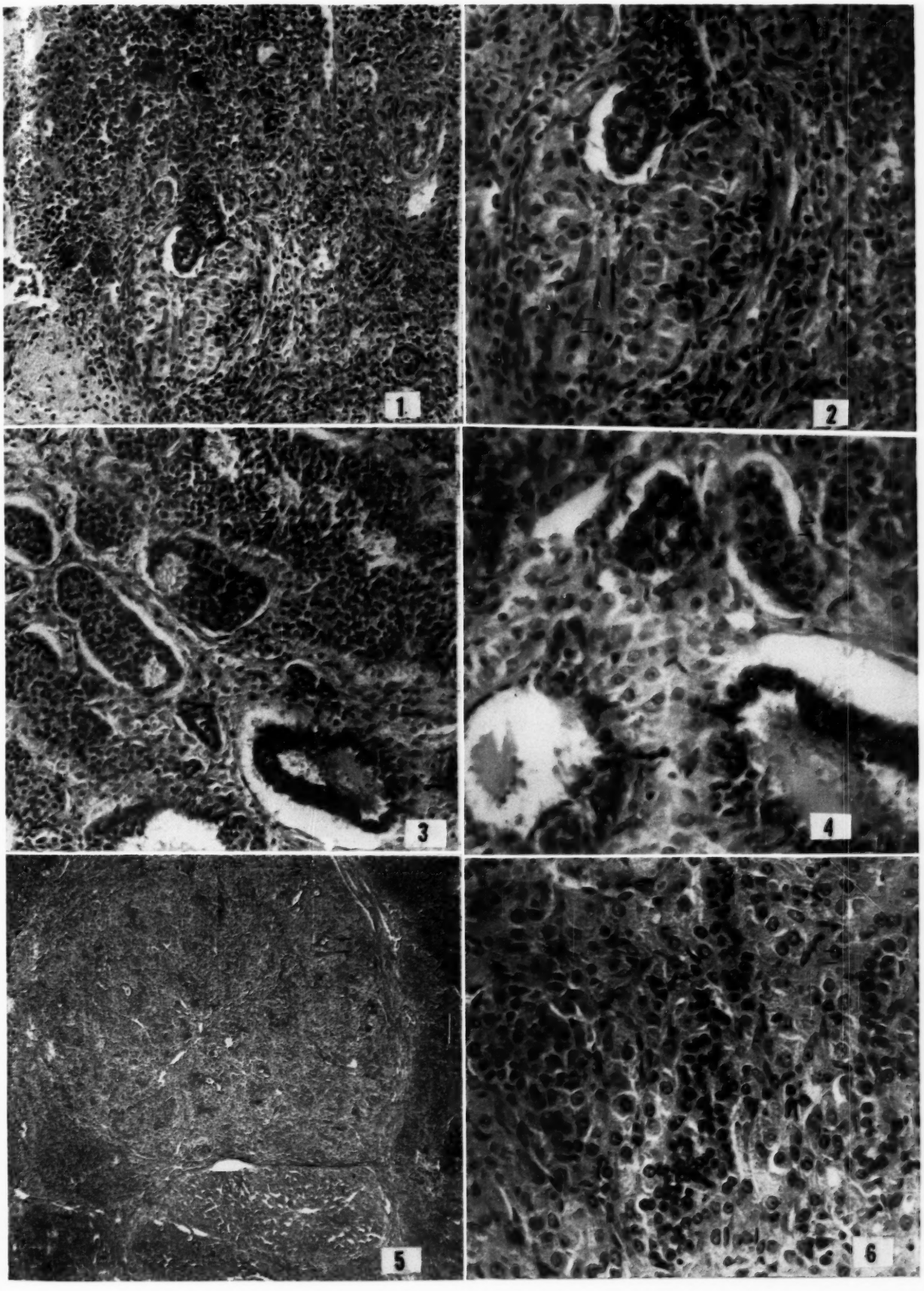
Fig. 2.—The corpus luteum and follicles of Fig. 1 are enlarged and show that both retain an essentially normal cytological pattern (×200).

Fig. 3.—Distinct clusters of granulosa-type cells are invading through a background of luteal tissue, in the typical pattern of a granulosa-cell tumor (×100). Castrated animal No. 3604 with intrasplenic ovary received luteotrophin for 175 days.

Fig. 4.—An enlargement of Fig. 3 shows granulosa cells arranged in dense clumps and tubular spaces in a background of luteal tissue (×250).

Fig. 5.—This transplant is unusual because an early granulosa-cell tumor is seen in the presence of well defined corpora lutea (×20). Castrated animal No. 3211 with intrasplenic ovary which received PMS for 126 days.

Fig. 6.—A zone of the granulosa-cell tumor in Fig. 5 is enlarged to show a background of luteal cells (×200). In this tumor the cords and clumps of granulosa cells differ in their arrangement from that noted in Figs. 2 and 3. The two tumors illustrate variations in cellular pattern that may occur.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Non-utilization of Radioactive Iodinated Uracil, Uridine, and Orotic Acid by Animal Tissues *in Vivo**

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Adenine (1, 6), guanine (1, 3, 5), cytidine (13), desoxycytidine (18), thymidine (18), and orotic acid (2), can be utilized by certain mammalian organisms for the synthesis of nucleic acids; and the rate of incorporation of many of these compounds into the nucleic acids of rapidly growing tissues, such as regenerating liver or neoplastic tissues, is greater than into those of resting tissues (10, 21).

Although 8-azaguanine is not a naturally occurring compound, evidence that it can be incorporated to a small extent into mammalian nucleic acids has been presented (16). This analog of guanine markedly inhibited the growth of *Tetrahymena geleii*, a guanine-requiring protozoan, and of certain experimental tumors. Kidder *et al.* (14) suggested that the carcinostatic properties of the analog might be explained on the basis of a requirement for guanine for the synthesis of nucleic acid by cancer cells. However, Mitchell and his coworkers (16) and Bennett *et al.* (4) were unable to demonstrate preferential utilization for nucleic acid synthesis of 8-azaguanine-2-C^{14} by a susceptible tumor (E 0771).

In view of the observations regarding the incorporation of 8-azaguanine into nucleic acids and the fact that there is a much greater rate of nucleic acid synthesis in tumor tissue than in the corresponding normal tissue of the same animal, this study was undertaken to investigate the possibility of a preferential concentration by tumor tissue of I^{131}-derivatives of naturally occurring pyrimidines. An incorporation of I^{131}-labeled pyrimidines could be of diagnostic and possibly of chemotherapeutic value. Considerable success has been attained in the use of I^{131}-labeled diiodofluorescein derivatives (17), iodinated human serum albumin (8), and sodium iodide (9) in the isotope-encephalometric localization of brain tumors. Furthermore, an I^{131}-labeled oxazine dye had a significant effect in prolonging the life of mice bearing transplanted tumors (19). If an effective and easily synthesized radioactive iodine-labeled compound could be found, the possibility might be afforded of the comparable use of compounds labeled with eka-iodine (astatine^{211}), a potent emitter of alpha particles, although this element is prepared with difficulty and has the inconveniently short half-life of 7.5 hours (12).

Three I^{131}-labeled pyrimidines, iodouridine-5-I^{131}, iodouracil-5-I^{131}, and iodoorotic acid-5-I^{131}, were synthesized, and their incorporation into normal tissue, regenerating liver, and certain experimental tumors was investigated. The results revealed that none of these compounds is incorporated to any significant degree into any of the tissues studied.

EXPERIMENTAL

Synthesis of iodoorotic acid-5-I^{131}.—To a solution of radioactive iodide (5 mc.; 1 mμg.; 0.8 ml.), contained in a 15 ml.-centrifuge tube, was added NaOH (10 per cent, 0.1 ml.) and carrier KI (4 mg.). The centrifuge tube was fitted with a stopper containing inlet and outlet tubes, and the solution was then concentrated to a volume of 0.2 ml. by passing a jet of dry air over it while it was heated on the steam bath. The exhaust air was led through a cold carbon tetrachloride trap and finally directly into the flue of the fume hood.

The concentrated solution was cooled in an ice bath, and the iodide was oxidized by the addition of H_2SO_4 (18 N, 0.2 ml.) and 0.005 M KIO_3 (0.3 ml.). A solution of orotic acid (200 mg.) in NaOH (3 N; 2 ml.) was added, and this was followed immediately by iodine (320 mg.). The mixture was heated on the steam bath for 15–20 minutes. During the heating, a further addition of NaOH (3 N) was made if necessary to effect complete solution of the iodine. The reaction mixture was cooled in ice; upon acidification with acetic acid a white

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precipitate of the sodium salt of 5-iodoorotic acid was formed. The free acid was formed upon recrystallization from 0.1 N HCl. The product was centrifuged off, and the supernate was discarded. In an attempt to remove any unreacted iodine, the precipitate was washed with 1:1 ethanol-acetic acid until the supernate was colorless, then once with ethanol and twice with ether. The iodoorotic acid was then recrystallized twice from 50 per cent ethanol.

With rapid heating the free acid decomposed at 270°–278° C. with the evolution of iodine vapors. In several syntheses the incorporation of radioactive iodine ranged from 40 to 50 per cent of the theoretical value. The elementary analysis[1] was: found: C, 21.37; H, 0.97; N, 10.14; I, 44.89; calculated: C, 21.28; H, 1.06; N, 9.94; I, 45.03. When the radioactive iodoorotic acid was chromatographed, using the *iso*-amyl alcohol-5 per cent Na_2HPO_4 system of Carter (7), the center of radioactivity, measured in a well-type scintillation counter, coincided with the ultraviolet absorbing spot R_F, 0.89, as located by a Mineralite ultraviolet lamp, model SL-2537. In NaOH (0.01 N), the ultraviolet absorption spectrum showed a maximum at 303 mμ (ϵ = 9,170) and a minimum at 255 mμ; and in HCl (0.01 N) a maximum at 287 mμ (ϵ = 6,460) and a minimum at 246 mμ were observed.

The position of the iodine atom has been assigned to carbon-5 for the following reasons. Hydroxy (oxy) groups are already attached to the carbon atoms at the 2 and 4 positions, and a carboxyl group is attached to carbon-6 (the new numbering system of Chemical Abstracts is used). If the iodine together with the carboxyl group was attached to the carbon at position-6, the typical ultraviolet absorption spectrum characteristic of a pyrimidine structure would have been destroyed because of inhibition of resonance caused by saturation of the 5–6 double bond. For a similar reason, the iodine together with a hydroxy group could not be attached to the carbon at either position-2 or -4. The possibility of substitution of either of the hydroxy groups at positions 2 and 4 is ruled out by the elementary analysis. The formation of a hydrogen iodide complex by orotic acid is not only highly improbable, because of the absence of any basic groups in the molecule, but, in addition, is also ruled out by the fact that the iodoorotic acid retained the theoretical amount of iodine after four recrystallizations from HCl (0.1 N). Therefore, the only available position left for substitution by iodine, which satisfies the spectral data and the elementary analysis, is position 5 of the pyrimidine ring.

Synthesis of iodouridine-I^{131}.—Radioactive iodide solution (10 mc.), as obtained from Oak Ridge, and a solution of KI (0.06 M, 0.3 ml.) were pipetted into a 25-ml. round-bottomed flask (glass-stoppered) and evaporated to 0.5 ml. by heating on a steam bath while passing a stream of air into the flask. After the addition of HNO_3 (8 N, 0.5 ml.) to oxidize the iodide to free iodine, water (25 ml.), uridine (200 mg.), iodine (200 mg.) and chloroform (2 ml.) were added, and the resultant mixture was refluxed on a steam bath for 8 hours. All operations were performed in the hood, and special precautions were taken to minimize contamination of the atmosphere with radioactive iodine.

All joints were sealed with silicone, and air was blown through the top of the condenser to carry any iodine vapors into a $CHCl_3$-trap which was cooled in an ice bath; emitted air was passed directly into the flue of a fume hood. During refluxing, clusters of long white needles of iodouridine were formed. After cooling overnight at 5° C., the chloroform and aqueous layers were discarded, and the remaining crystals of iodouridine were washed with ether until the solvent was colorless. The yield at this point was 200 mg., with 32 per cent of the original I^{131} incorporated or 64 per cent of theory. The compound was further purified by treatment with Nuchar (C250) and by recrystallization from hot water.

With rapid heating the compound decomposed at 205°–208° C. with the evolution of iodine vapors. The point of decomposition varied with the rate of heating. In NaOH (0.01 N) the ultraviolet absorption spectrum showed a maximum at 278 mμ and a minimum at 253 mμ, and in HCl (0.01 N) a maximum at 289 mμ and a minimum at 249 mμ were observed. The elementary analysis[1] was: found: C, 29.17, 29.14; H, 2.95, 3.03; N, 7.48, 7.53; I, 34.09, 34.18; calculated: C, 29.10; H, 3.24; N, 7.50; I, 34.33.

Further evidence that this compound is 5-iodouridine was given by its reaction to bromine water; whereas addition of the latter to an aqueous solution of uridine resulted in immediate decolorization of the bromine water, one drop of this reagent retained its color when added to an aqueous solution of iodouridine. Hydrolysis of iodouridine with $HClO_4$ (12 N) in a boiling water bath for 1 hour resulted in a compound having an R_F similar to that of uracil in two solvent systems (7, 20), as indicated in Table 1. Violet fumes, indicating a cleavage of the organic iodine, were formed during the hydrolysis. Silver nitrate added to the acidified (HNO_3) aqueous solution of iodouridine gave no

[1] The elementary analyses were performed by Dr. E. W. D. Huffman, Denver, Colo.

precipitate, an indication that the iodine atom was organically bound.

Synthesis of iodouracil-I^{131}.—The method used was a modification of that of Johnson and Johns (11). Radioactive iodide solution (5 mc.) was pipetted into a 50-ml. centrifuge tube and placed in an ice bath. A solution of KI (0.06 M, 0.1 ml.) was added, and then H_2SO_4 (18 N, 0.05 ml.) and a solution of KIO_3 (0.005 M, 0.3 ml.) in order to oxidize the iodide to free iodine. After the addition of a solution of uracil (200 mg.) in NaOH (2.5 N, 1.4 ml.), an I_2-KI solution was added until a definite excess of free iodine was present. Upon warming and adding additional I_2-KI solution to maintain an excess of iodine, iodouracil began to crystallize. The suspension was cooled in an ice bath for 1 hour, and then the crystallized iodouracil was removed by centrifugation. The residue was suspended in water, acidified with acetic acid (9 N), and again centrifuged. The residue was washed with acidified alcohol and then 5 times with boiling ether. The compound was recrystallized from water and then from alcohol. The yield was 276 mg. of iodouracil with 32 per cent incorporation of the original I^{131} added or 64 per cent of the theoretical value.

Determination of radioactivity.[2]—The concentration of I^{131} in the tissues studied was determined with the aid of a scintillation counter constructed by MacIntyre (15). Since I^{131} emits highly penetrating gamma radiations, the tissue concentration of I^{131} could be determined directly without fractionation or isolation. In the earlier studies each tissue was dissolved in NaOH (6 N) to give a final volume of 5 ml. in a calibrated test tube. With a specially constructed test tube holder, to maintain constant geometrical relationships, the radioactivity was determined by comparing it to the same volume of a radioactive iodide solution of known specific activity. In order to eliminate the necessity of dissolving the tissue and also to improve the geometric advantage, tissues were examined subsequently by placing individual samples in a planchet and locating this directly beneath the crystal or the scintillation counter.

RESULTS AND DISCUSSION

The results obtained from injections of iodoorotic acid, iodouridine, and iodouracil labeled with iodine-131 into various animal species, normal and otherwise, as indicated, are shown in Table 2. No preferential incorporation of these iodinated pyrimidine derivatives into regenerating liver or experimental tumors was observed; the tumors employed were Sarcoma 180, Brown-Pearce carcinoma, adenocarcinoma E 0755, 4-dimethylaminoazobenzene-induced hepatoma, and an x-ray-induced bone tumor.[3] Both thyroid and gastric tissues usually contained a higher concentration of radioactive iodine than did the other tissues which were investigated. Since these tissues are known to concentrate the iodide ion, the probability of the partial hydrolysis of these organic iodo-compounds *in vivo* is indicated. Tumors occasionally had some activity, but never more than that of other tissues. The values shown for the distribution of radioactivity among the various tissues were obtained as part of a screening program; hence, because of the limited number of animals studied, these results should not be interpreted as being a statistically acceptable tissue distribution.

TABLE 1

THE R_F VALUES OF HYDROLYTIC PRODUCTS OF SOME IODINATED PYRIMIDINES

	Iso-amyl alcohol-5 per cent Na_2HPO_4		*n*-Butanol saturated with HCl (0.1 N)	
Compound	R_F before hydrolysis	R_F after hydrolysis*	R_F before hydrolysis	R_F after hydrolysis*
Iodouracil	0.52	0.52	0.55	0.60
Uracil	0.69	0.72	0.32	0.32
Iodouridine	0.70	0.69	0.34	0.32
Uridine	0.80	0.71	0.18	0.34

* With 12 N $HClO_4$ for 1 hr. in boiling water bath.

Prior to the administration of iodoorotic acid, an investigation was conducted of its toxicity in white mice. Eighteen mice in groups of three each were given intraperitoneal injections of $NaHCO_3$ (1 per cent, 0.2 ml.) containing 0, 1, 5, 10, 15, or 20 mg. of iodoorotic acid. No toxic effect was observed in any of the animals.

Radioactive iodine derivatives of orotic acid, uridine, and uracil do not appear to be of diagnostic value for the localization of centers of rapid nucleic acid synthesis, and, under the conditions of these experiments, no chemotherapeutic activity was evidenced.

SUMMARY

Iodouridine, iodoorotic acid, and iodouracil, labeled with I^{131}, were synthesized. The syntheses of

[2] We are grateful to Dr. H. L. Friedell, Dr. W. J. MacIntyre, and Mr. J. S. Krohmer for advice and assistance in the measurements of radioactivity and for supplying the I^{131}, allocated to them by the Atomic Energy Commission.

[3] We are grateful to Dr. Harold P. Rusch of the McArdle Memorial Laboratory of the University of Wisconsin for rats bearing 4-dimethylaminoazobenzene-induced hepatoma and mice carrying Sarcoma 180; to Dr. Alfred Gellhorn of Columbia University for donor rabbits bearing the Brown-Pearce carcinoma in the anterior chamber of the eye and donor mice bearing adenocarcinoma E 0755; and to Dr. Simon Koletsky of the Atomic Energy Research Project and the Department of Pathology of this School of Medicine for making available a rat bearing an x-ray-induced bone tumor.

TABLE 2

ADMINISTRATION AND LOCALIZATION OF RADIOACTIVE IODINE DERIVATIVES OF NATURALLY OCCURRING PYRAMIDINES

COMPOUND AND SOLVENT	ANIMAL	CONDITION	METHOD OF ADMINISTRATION	TOTAL DOSE	TIME OF SACRIFICE	CONCENTRATION OF ACTIVITY	
						Tissue	Microcuries per gram wet weight
5-Iodouridine-I^{131} (in 2 per cent $NaHCO_3$)	Rat (1)*	Partially hepatectomized	IP†	100 μc. (6.25 mg.) in 5 divided doses at 2-hr. intervals starting 18 hr. after hepatectomy	16 hr. after last injection	Thyroid Stomach Remainder	0.46±0.023 0.01±0.001 <0.005
"	Mouse (2)	Sarcoma 180	IP	65 μc. (4.10 mg.) in 5 divided doses during a 48-hr. period	24 hr. after last injection	Thyroid and trachea Stomach Tumor Liver Muscle	0.054±0.03; 0.90±0.09 1.10±0.05; 0.10±0.01 0.25±0.01; 0.13±0.01 0.04±0.002; 0.01±0.005 0.06±0.003; 0.01±0.005
"	Rabbit (2)	Anterior chamber-transplant of Brown-Pearce carcinoma	IV‡	750 μc. (47.0 mg.) in 5 divided doses over a 48-hr. period	"	Thyroid Tumor Stomach	6.4±0.32 <0.005 0.89±0.045
5-Iodouracil-I^{131} (in propylene glycol)	Rat (1)	Normal	IP	10 μc. (1.73 mg.)	48 hr. after injection	All tissues	<0.005
"	"	X-ray-induced bone tumor	Jugular vein	25 μc. (4.3 mg.)	24 hr. after injection	All tissues	<0.005
"	Rat (2)	4-Dimethylaminoazobenzene induced hepatoma	IV	60 μc. (10.5 mg.) in 3 divided doses at 24-hr. intervals	48 hr. after last injection	Thyroid Tumor Stomach Liver Spleen Remainder	0.90±0.09; 0.25±0.13 0.01±0.005; 0.06±0.01 0.03±0.01; 0.03±0.01 <0.005; 0.03±0.006 <0.005; 0.03±0.015 <0.005; <0.005
"	Rat (2)	"	"	150 μc. (25.0 mg.) in 3 divided doses at 24-hr. intervals	"	Liver Tumor Thyroid Stomach Remainder	0.25±0.13; <0.005 1.36±0.06; 0.14±0.07 1.64±0.16; 3.52±0.07 2.16±0.02; 0.02±0.01 0.25±0.13; <0.005
5-Iodouracil-I^{131} (in 1 per cent Na_2CO_3)	Mouse (1)	Adenocarcinoma E 0755	IP	20 μc. (2.46 mg.) in 2 divided doses at 24-hr. intervals	3 hr. after last injection	Thyroid and trachea Tumor Testicle Liver Muscle	0.27±0.14 0.15±0.015 0.31±0.03 0.04±0.004 0.27±0.003
"	"	"	"	5 μc. (0.86 mg.)	2 hr. after injection	Thyroid Tumor Testicle Spleen Stomach	0.14±0.07 0.10±0.01 0.07±0.007 0.08±0.04 0.02±0.01
"	Rabbit (1)	Brown-Pearce carcinoma	IV	50 μc. (8.6 mg.)	24 hr. after injection	Thyroid Remainder	0.34±0.03 <0.005
5-Iodoorotic acid-I^{131} (in 1 per cent $NaHCO_3$)	Rat (2)	Partially hepatectomized	IP	96 μc. (14.4 mg.) in 3 divided doses at 4-hr. intervals per day, for 2 days, starting 24 hr. after partial hepatectomy	19 hr. after last injection	Thyroid Stomach Remainder	0.90±0.30; 0.52±0.025 <0.005; 0.03±0.015 <0.005; <0.005
"	Mouse (3)	Sarcoma 180	IP	10 μc. (1.5 mg.)	3, 6 and 24 hr. after last injection	3-hr. tissues Thyroid and trachea Liver Tumor Muscle Bone marrow Remainder 6 and 24-hr. tissue	 0.10±0.05 0.01±0.005 0.03±0.015 0.08±0.008 0.38±0.038 <0.005 <0.005

TABLE 2—*Continued*

Compound and solvent	Animal	Condition	Method of administration	Total dose	Time of sacrifice	Concentration of activity	
						Tissue	Microcuries per gram wet weight
"	"	Adenocarcinoma E 0755	"	"	"	3-hr. tissues	
						Thyroid and trachea	0.16±0.08
						Tumor	0.08±0.04
						Muscle	0.05±0.005
						Bone marrow	0.07±0.02
						Remainder	<0.005
						6 and 24-hr. tissue	<0.005
"	Rabbit (1)	Anterior chamber-transplant of Brown-Pearce carcinoma	IV	800 μc. (120 mg.) in 4 divided doses at 24-hr. intervals	24 hr. after last injection	Thyroid	0.24±0.003
						Eye tumor	<0.005
						Remainder	<0.005

* Number of animals.
† IP = intraperitoneal injection.
‡ IV = intravenous injection.

iodouridine and iodoorotic acid have not been described previously. The incorporation of these three I^{131}-labeled pyrimidines into normal tissue, regenerating liver, and experimental tumors was investigated, and no significant preferential concentration was observed.

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Studies on the Biological Action of Malononitriles*

III. Effect of Malononitrile Administration on Excretion of Thiocyanate in Normal and Walker Carcinoma 256-bearing Rats

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In a previous study it was observed that certain substituted malononitriles produced retardation of the growth of certain transplanted tumors in mice (6). An explanation for the retarding effect of these compounds on tumor growth would be of great value in establishing a foundation for a logical approach to the problem of tumor chemotherapy.

The study of the excretion of thiocyanate was undertaken for this purpose with the following aims in mind: (*a*) To determine more quantitatively if any relationship existed between the amount and rate of release of cyanide from the malononitriles and their effectiveness. In the previous investigation no such relationship was apparent (6). (*b*) To secure comparative information on the fate of a variety of nitriles and thus some insight into the manner in which the different nitriles are metabolized. (*c*) To determine if malignant growth affected the cyanide metabolism of the organism (12) and thereby altered the pattern of the detoxication mechanism of the nitriles by the body. The discovery of such differences in metabolic patterns might perhaps yield additional information on the effects of malignancy on the metabolism of the tumor-bearing host and contain diagnostic implications. These features of the problem were added incentives in undertaking this investigation even though the present knowledge regarding the fate of nitriles in the normal animal is extremely sketchy.

MATERIALS AND METHODS

Sprague-Dawley and Long-Evans rats of both sexes weighing between 250 and 350 gm. were employed. No significant differences were observed between the two strains. In the experiments with the nitriles only males were used. The animals were kept individually in wire screen-bottomed metabolic cages fitted with large glass funnels to collect the urine. The animals were trained to eat daily between 10–12 A.M. for 3 days before the experiment was started. On the fourth day they were weighed, fed, and then injected. They were then returned to the metabolic cages which were now fitted with fine mesh wire gauze over the funnels so as to prevent solid particles from getting in the urine. Each funnel also carried a fine porcelain plate to serve as a filter. The cages were cleaned daily.

The animals received weighed amounts of powdered laboratory Purina Chow which was moistened to minimize scattering, while water was allowed ad libitum. The daily endogenous thiocyanate[1] values were determined over a period of 6 days, after which interval the same animals were given injections of a single dose of one of the test compounds in 1 ml. of solvent. The urine was preserved under toluene and collected daily for another 6 days. The volume and pH (at 25° C.)[2] of the daily samples were recorded, and the samples were analyzed individually for their thiocyanate content by a spectrophotometric method based on the absorption of ferric thiocyanate at 520 mμ.

Experiments on the Walker carcinoma-bearing rats were started after permitting the tumor transplants to grow for a period of 2 weeks.

Method of thiocyanate determination.—The urinary thiocyanate content was determined by a

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[1] The values of the normal daily thiocyanate excretion served as "background" values and these, being related to the metabolism of normally occurring cyanide, have been designated as *endogenous*, in contrast to the *exogenous* cyanide obtained after nitrile administration.

[2] The method of urine collection made the carbon dioxide loss unavoidable.

combination and modification of the methods of Brodie and Friedman (4) and Chesley (5).

The procedure finally adopted is as follows: 5 ml. of urine was pipetted into a 100-ml. beaker and carefully adjusted to pH 10.5–10.8 by adding, dropwise, 10 per cent KOH solution. (If the total volume was less than 5 ml., it was made up to that volume with distilled water.) One gm. of charcoal (Norite B) was added, and the urine was boiled for a few seconds and filtered (centrifuging at this point is more effective).[3] The charcoal residue was washed with four 5-ml. portions of water. The supernatants were pooled and acidified with 0.5 ml. of 4 N nitric acid. The solution was then treated with 5 ml. of ferric nitrate-nitric acid color reagent (prepared by dissolving 50 gm. of $Fe[NO_2]_3 \cdot 9H_2O$ in about 800 ml. distilled water, by adding 25 ml. concentrated nitric acid, and by making the volume up to exactly 1 liter). The sample was then made up to a volume of 30 ml., thoroughly mixed, and put aside for 15 minutes in a dark place. An aliquot was then read in the spectrophotometer. The spectrophotometer readings were converted to thiocyanate values by using a standard curve in which the absorption of solutions containing 2 to 1,000 μg of thiocyanate/5 ml was plotted against concentration. Results are expressed in micromoles (μM) of thiocyanate unless otherwise stated.

The samples were treated with charcoal to remove the pigments in the urine prior to forming the colored iron-thiocyanate complex. This treatment was found to adsorb some thiocyanate from both the urine samples and the standard solutions employed in making the calibration curve. The adsorption of thiocyanate by charcoal has been previously reported (11), and it was difficult to understand the observation of Brodie and Friedman (4) who reported 90–100 per cent recoveries of thiocyanate from tissues after decolorization with charcoal. To equalize the experimental conditions, the standard solutions, adjusted to pH 10.5, were treated with charcoal in the same way as the urine, and a correction was calculated for determining the thiocyanate contents of the urine samples prior to charcoal treatment.

The nitriles used in these experiments and the method of establishing their toxicity were previously described (6).

RESULTS AND DISCUSSION

This paper reports only the results obtained with nitriles that retarded tumor growth, their parent compounds, and sodium cyanide—the latter to establish the excretion pattern of the cyanide ion.

Endogenous excretion of thiocyanate.—Calculation of the thiocyanate in the urine which was derived from the different compounds administered required that the endogenous thiocyanate excretion be subtracted from the total thiocyanate excretion. The determination of this value proved to be difficult because of the great individual variations in the endogenous thiocyanate excretion even under carefully controlled conditions of food intake, weight, and sex. The variations, possibly, may be a reflection of the differences in the rate of the protein metabolism of the individual animals. This difficulty was minimized by using a large number of animals and by determining the average daily thiocyanate excretion in normal and tumor-bearing animals prior to their treatment with the nitriles. In establishing the value of the endogenous thiocyanate excretion, consideration was given to the vehicles employed for injection of the nitriles, namely, water and asymmetrical propylene glycol. The data obtained for the vehicles employed are given in Table 1.

To permit analysis of the frequency distribution, the individual values, which ranged between 0.8 and 4.5 μM per day, were arranged in class intervals of 0.4 μM (10 μg. CN^-).[4] These results are plotted in Chart 1. The frequency distribution revealed no definite "trends."

The plot of the frequency distribution of the data revealed that this distribution was skewed, in which case it is customary to regard the mode rather than the arithmetical mean as the true mean. From Chart 1 it is seen that the mode of the frequency distribution of group A male rats and that of the tumor-bearing rats of group B was 2.5 μM (65 μg.), whereas that of the normals of group B was 1.6 μM (40 μg.) and that of the tumor-bearing animals of group A was 2.7 μM (70 μg.). On the basis of these observations it is reasonable to assume that there is no real difference in the endogenous thiocyanate excretion between normal and tumor-bearing animals.[5]

The average daily endogenous thiocyanate excretion was calculated to be 2.3 μM SCN^- for rats of either sex between the weights of 250 and 350

[3] Tumors occasionally break through the skin, and thus blood gets into the urine. This can be recognized by the white precipitate formed upon boiling the urine with charcoal. In such samples filtration does not suffice, and the gray pigment can only be removed by centrifugation.

[4] In order to retain uniformity of presentation with other publications of the authors, the values in the chart are given as cyanide equivalents.

[5] This result is contrary to an earlier preliminary report by the authors (8) which appeared to show a lower endogenous excretion by the tumor-bearing than by the normal rats. In view of the range of variation found in the present, more extensive series, the earlier data were thus not sufficiently conclusive.

TABLE 1

THE ENDOGENOUS URINARY THIOCYANATE EXCRETION BY NORMAL AND WALKER CARCINOMA-BEARING RATS*

Group	pH of urine	Urine flow ml/hr/100 gm body wt	SCN^-/ml/day μM	SCN^-/day μM	No. determinations
Males:					
Normal†	7.1	0.33	0.250	2.58±0.72	55
Tumor-bearing†	6.6	0.29	0.240	2.15±0.99	52
Normal‡	7.4	0.30	0.235	2.15±1.09	73
Tumor-bearing‡	6.9	0.28	0.255	2.19±0.82	45
Females:					
Normal†	7.9	0.25	0.250	1.96±0.59	54
Tumor-bearing†	7.6	0.30	0.245	2.23±0.76	50
Normal‡	7.6	0.20	0.320	1.92±0.66	60
Tumor-bearing‡	7.8	0.38	0.195	2.27±0.63	50

* Values are given as calculated for average of 300 gm wt. animals.
† Animals injected with propylene glycol.
‡ Animals injected with water.

gm. This value, therefore, was substracted from the daily excretion values found for the urine of the nitrile-treated animals.

Exogenous thiocyanate excretion from nitriles.—The daily exogenous urinary thiocyanate was calculated from the following relationship; $X\mu M = av/5 - 2.3$, where a is the amount of thiocyanate, in μM/ml, corrected for the adsorption by charcoal, and v is the daily urine volume collected.

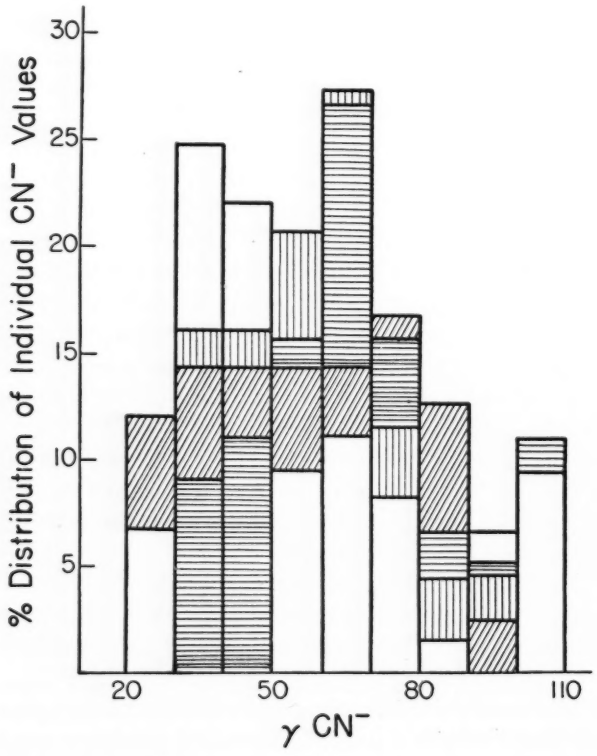

CHART 1.—Frequency distribution of daily endogenous thiocyanate excretion.

□ = Normal control (water).
▥ = Normal control (propylene glycol).
▤ = Tumor-bearing control (water).
▨ = Tumor-bearing control (propylene glycol).

Table 2 shows the extent of the conversion of nitrile groups to SCN^- over the 6-day collection period. Most of the nitriles had to be administered in asym. propylene glycol because of their insolubility in water. The difference between the two vehicles can be estimated from the results obtained with the parent compound, malononitrile, in both vehicles. A lower degree of the conversion to SCN^- was observed when this nitrile was administered in propylene glycol. It is also to be noted that, in general, the excretion values were lower in the Walker carcinoma-bearing rats. While the difference in the amounts excreted between the normal and tumor-bearing animals was not statistically significant, the consistency of the low values in the tumor-bearing animals may possibly represent a trend. This must be viewed with caution, as it is not known how much the lower values may be due to the continued increment in tissue and body fluid volume caused by the continuous growth of the highly vascularized Walker carcinoma. The lower excretion values could reflect the diversion of thiocyanate into the additional extracellular fluid formed.

Effect of nitro group on the amount and rate of thiocyanate excretion.—The experimental data contained in Table 2 substantiate the previous contention (6) that the release of cyanide was not responsible for the growth-retarding effect of the nitro-substituted malononitriles. This also explains why thiosulfate was ineffective in reducing their toxicity. The effect of nitro substitution in lowering or suppressing the conversion of nitriles to thiocyanate was also reported by Adeline *et al.* (1), who fed *p*-nitrophenyl- and *p*-nitrobenzylcyanide to rabbits. That report contains only qualitative estimates of the amounts of thiocyanate excreted after their administration. Earlier experiments with aromatic nitriles administered to dogs led to

contradictory results (2, 9). The effect of the nitro group in decreasing the amount of cyanide released was also observed with the malononitriles. The decrease in the rate of the release of cyanide may be due either to the structural effect of the nitro group in enhancing the chemical stability of the nitrile group, or, alternatively, to a decreased ability of these compounds to combine with the enzyme that cleaves the cyanide from the molecule, if the cyanide production is an enzymatic process.

These differences in the stability of the nitrile group caused earlier authors (1) to postulate that the toxicity of the nitriles was a highly specific property of the molecule rather than a function of the rate at which they split off cyanide.

That nitro substitution does influence the rate at which cyanide is released from nitriles is demonstrated in Chart 2. There it is shown that, whereas the elimination of cyanide is highest in the first day for malononitrile and is virtually completed in 3 days, the peak of elimination with *p*-nitrobenzalmalononitrile is on the third day. The results show an obvious shift in the maximum of the thiocyanate excretion in the *p*-nitrobenzalmalononitrile-treated animals. It is relevant to the significance of the effect of nitro substitution that no such shift in excretion time was found with benzalmalononitrile either in normal or in tumor-bearing animals (7).

The experiments illustrated in Chart 2 were carried out primarily to study the effect of malignancy on the removal of the nitrile group. The results show that there is no difference either in the rate of thiocyanate excretion or in the percental conversion of nitrile groups to cyanide, associated with the condition of malignancy.[6]

It was assumed that a period of 6 days was sufficiently long to enable complete resorption and distribution of any nitrile from the peritoneal cavity, no matter how poor the solubility was. The thiocyanate excretion, without exception, decreased to normal by the sixth day.

The daily reading of the pH values of the urine demonstrated that no particular pathological involvement of the kidneys took place after the administration of the nitriles.[7]

TABLE 2

URINARY THIOCYANATE EXCRETION BY NORMAL AND WALKER CARCINOMA-BEARING RATS FOLLOWING ADMINISTRATION OF MALONONITRILES*

	Group		No. animals	pH of urine	Urine flow ml/hr/100 gm body wt	CN^- equiv. administered μM†	SCN^- excreted in 6 days μM	Per cent conversion	Carcinostatic effect‡
A.	Sodium cyanide	N	15	7.7	0.27	20.4	11.9±6.8	58	−
		T.b.	10	6.9	0.21		11.2±3.65	55	
	Malononitrile	N	10	6.9	0.32	151	33.5±8.7	22	−
		T.b.	10	6.7	0.26		25.8±5.2	17	
	Ethoxymethylene-malononitrile	N	15	7.2	0.31	81.6	21.2±8.7	26	±
		T.b.	10	6.9	0.25		18.0±7.2	22	
B.	Malononitrile	N	15	8.6	0.31	151	24.2±8.8	16	−
		T.b.	10	7.5	0.22		19.6±8.85	13	
	Benzalmalononitrile	N	6	7.4	0.29	65	13.0±3.85	20	−
		T.b.	6	7.5	0.23		7.8±3.4	12	
	p-Nitrobenzal-malononitrile	N	10	8.0	0.27	50	2.5±1.5	5	++
		T.b.	10	6.5	0.23		1.5±0.45	3	
	Furanalmalononitrile	N	8	7.5	0.23	75	9.7±6.5	13	−
		T.b.	6	7.5	0.42		6.0±3.9	8	
	5-Nitrofuranalmalononitrile	N	8	8.0	0.37	26§	1.8±1.7	7	++
		T.b.	8	6.4	0.25		<0.2	<1	

N = normal; T.b. = tumor-bearing.
Group A: vehicle—water.
Group B: vehicle—asym. propylene glycol.
* SCN^- values are for average of 300-gm. animals (the animals lost an av. of 20–30 gm. within 6 days).
† Corresponds to 5 mg. of nitrile njected.
‡ See reference (7) for explanation of symbols.
§ Corresponds to 2.5 mg. of nitrile injected.

An interpretation of the biological action of the malononitriles has been considered in the light of the urinary thiocyanate levels following the administration of the malononitriles. The authors are aware that such an analysis does not explain the fate of the nitriles within the body and leaves many questions unanswered. Nevertheless, the urinary thiocyanate excretion affords some meas-

[6] It was interesting to note that no significant increase in the thiocyanate level was found in the blood of human beings after the administration of the nitro-substituted malononitriles (10). This seems to give an added weight to our observations that the release of cyanide is not the property of the nitriles requisite for their effect in retarding tumor growth.

[7] The delayed toxic effect of 5-nitrofuranalmalononitrile is notable in both normal and tumor-bearing animals. A single dose of 2.5 mg/ml in propylene glycol killed 80 per cent of the animals by the sixth day.

ure of comparison between the amount of nitrile introduced and the amount converted into thiocyanate.

SUMMARY

1. No statistically significant difference was noted between the daily excretion of endogenous thiocyanate by normal and by tumor-bearing animals. Neither sex nor the injection of water or propylene glycol (vehicles used for the administration of nitriles) had any influence on this excretion. The statistically established average value for endogenous thiocyanate excretion was 2.3 μM/day for the rat. There is evidence for continuous cyanide formation in the body as a part of the general metabolism.

2. The daily excretion of thiocyanate for 6 days following intraperitoneal administration of single doses of various malononitriles displayed a "trend" toward lower values in the tumor-bearing animals.

3. In confirmation of a previous report (6), the experimental data showed that the activity of malononitriles in retarding tumor growth was not due to release of cyanide, as the percentage conversion of these compounds to cyanide was much smaller than that of the malononitriles without tumor-inhibitory action.

4. The study of the rate of thiocyanate excretion showed that, as compared to malononitrile and benzalmalononitrile, nitro substitution produced a shift in the time of maximum excretion of thiocyanate. The selectivity of the nitro group in causing this shift is presumptive as no other ring substitutions were investigated.

5. No significant changes in the pH of the urine were caused by administration of single doses of nitriles.

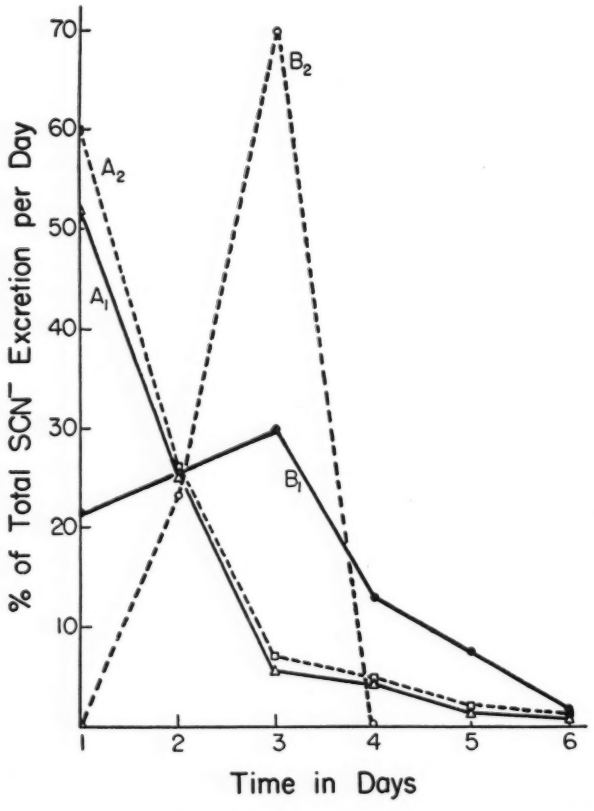

CHART 2.—The rate of SCN^- excretion by normal and tumor-bearing rats after malononitrile and *p*-nitrobenzalmalononitrile administration.

A_1 = Normal rat treated with malononitrile.
A_2 = Tumor-bearing rat treated with malononitrile.
B_1 = Normal rat treated with *p*-nitrobenzalmalononitrile.
B_2 = Tumor-bearing rat treated with *p*-nitrobenzalmalononitrile.

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Adrenal Tumors and Other Pathological Changes in Reciprocal Crosses in Mice

II. An Introduction to Results of Four Reciprocal Crosses*

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This report is to serve as an introduction to hybridization studies which have been undertaken concerning adrenal cortical tumors and related pathological conditions. It is planned that reports similar to that for the reciprocal DBA and CE hybrids (27) will also be made.

Hybridization studies have been used in many types of experiments with animals and with plants. Such experiments were of the type Mendel used and on which he based his principles of unit inheritance. Hybridization experiments with animals can be used not only to determine chromosomal inheritance, but also for purposes of evaluating other types of influences on the offspring, e.g., maternal influence influential in breast tumor occurrence in mice.

The effects of gonadectomy on certain inbred strains of mice have already been reported in detail (9, 10, 19, 23, 28–36), as well as some intensive studies on the pituitary (3, 5, 26) and the uterus (2) of some F_1 reciprocal crosses. Recently, the findings on the reciprocal cross of strains DBA and CE have been reported (27). With this as a pattern, it is possible to indicate how the other reciprocal crosses thus far investigated compare with this series.

Primarily, the entire series was undertaken to determine the effect of inheritance on the response of the adrenals of the F_1 animals to gonadectomy. However, certain other factors could not be neglected and have been included in the results. A brief review of a few of the characteristics of the inbred strains used as parental strains in these reciprocal crosses may help to evaluate the results obtained in this now extensive pilot experiment.

It has been customary to characterize inbred strains according to certain factors, e.g., mammary tumor incidence, susceptibility or resistance to the mammary tumor inciter (MTI), response to gonadectomy, and incidence of various other types of tumors. Some of these characteristics will be briefly considered, where possible, for the inbred parental strains used. (*a*) Strain DBA has a moderate mammary tumor incidence; following gonadectomy, nodular hyperplasia of the adrenal cortex develops, and the accessory reproductive organs become feminized (10, 28, 29, 30). (*b*) Strain CE has no mammary tumors but is thought to be susceptible to the MTI. Old females in this strain sometimes develop granulosa-cell tumors of the ovary. Gonadectomy causes adrenal cortical carcinomas to develop, and concomitant with the tumor is feminization and/or masculinization of the accessory reproductive organs (31–35). (*c*) Strain C57BL/10 does not have mammary tumors and is relatively resistant to the MTI. Gonadectomy causes the "classical" castrate picture, no major changes in the adrenals, and the accessory reproductive organs typically remain small and immature. (*d*) Strain A/Wy has a low mammary tumor incidence in virgin females but a high (85–90 per cent) incidence in breeding females (17). The response to gonadectomy in this strain is very similar to the response of strain C57BL/10.[1] (*e*) In strain C3H, the mammary tumor incidence is high (93–96 per cent) in both virgin and breeding females.[1] The adrenal response to gonadectomy is similar to that of strain DBA.[1] (*f*) The yellow strain was introduced into the experiment because of its interesting genetic constitution and its dem-

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[1] Unpublished data.

onstrated ability to modify the incidence of mammary and lung tumors. Homozygous yellow animals die early in embryonic life; therefore, all yellow animals are heterozygotes, and matings to these yellows produce two classes of offspring that, phenotypically at least, differ from each other by only one gene. It is known that yellow outcross animals often become relatively obese compared to other animals. The response to gonadectomy is not known.

MATERIALS AND METHODS

The experimental methods may be divided into two sections: (*a*) the manner in which the crosses were made and (*b*) the actual experimental procedure used for collecting data on the animals raised.

a) Five series of crosses were made as follows:

1. DBA/2Wy♀ × C57BL/10♂ and the reciprocal C57BL/10♀ × DBA/2♂.
2. DBA/2♀ × CE/Wy♂ and the reciprocal CE♀ × DBA/2♂.
3. CE♀ × C57BL/10♂ and the reciprocal C57BL/10♀ × CE♂.
4. A/Wy♀ × C3H/Jax♂ and the reciprocal C3H♀ × A♂.
5. YEL♀ × CE♂ which produced two types of F_1 offspring, yellow and agouti. No reciprocal cross was made.

In designating crosses hereafter in the text, the female parent is always listed first.

b) The experimental procedure differs very little from that used in previous studies on the response of various types of mice to gonadectomy. Animals were gonadectomized at birth to 3 days of age and then allowed to mature and age without further treatment. Intact virgin females and unmated males were raised as controls and aged with no treatment. All animals, experiment and control, were maintained under uniform environmental conditions and fed Purina Fox Chow and water ad libitum.

One of each of the intact females, males, gonadectomized females, and males was autopsied at 15 days, 1 month, and at monthly intervals up to 24 months of age and beyond to obtain a developmental series. An exception to this was the A × C3H and C3H × A set of crosses which were autopsied at bimonthly intervals beginning at 2 months of age. There were several gaps in the YEL × CE series that were not filled, because the animals were killed in the fire of 1947 and the cross was not started again.

Tissues regularly observed and saved for histological study were the submaxillary gland, both adrenals (which were serial sectioned), the ovaries (the left was serial sectioned) or testis (when present), uterus, vagina, the accessory sex organs (including seminal vesicles and prostates) of the males, the thyroid, and the pituitary. Other organs with any observed gross abnormalities were also saved. All tissues were ordinarily fixed in Tellyesnicky's fluid, cut at 8 μ and stained with hematoxylin and aqueous eosin. The pituitary glands, when abnormal, were fixed in modified Zenker's solution, serially sectioned at 4 μ, and differentially stained. The skins of all animals were fixed and saved so that the mammary glands could be studied.

Measurements on all tissues, where indicated, were made with an ocular micrometer as described previously (27). Statistical analyses, the significance of the mean difference of these various measurements, were determined by Fisher's method (11) for small samples using available paired observations.

RESULTS

GENERAL COMMENTS ON THE ANIMALS OF THE VARIOUS CROSSES

Both body size and activity separated the F_1 animals into two main groups: those in which CE is one of the parental strains and those without any CE inheritance .

DBA × C57BL/10 and the reciprocal F_1 animals did not seem much larger in body size than strain C57BL/10 animals but were larger than strain DBA/2 mice. As a whole, C57BL × DBA and the reciprocal animals were only moderately active and not skittish or nervous.

A × C3H and the reciprocals were fairly short of body, i.e., they did not appear to have so great a body length as any other F_1 animals. In activity they were the most calm and quiescent of all the F_1 animals raised.

On the other hand, DBA × CE and the reciprocal were long and rangy and the most active of all the F_1 animals. CE × C57BL and the reciprocals, although somewhat calmer, were large and rangy like the other CE hybrids.

The yellow animals became moderately obese and were very docile, while their nonyellow sibs were very similar to the other CE hybrids in size and activity.

Some indication of these size differences may be ascertained by comparing body weights of the animals of the various F_1 groups (Tables 1 and 2).

ADRENALS

Intact Animals

Size.—The adrenals of all intact animals were measured, and total adrenal, cortical, and medullary size were computed. These measurements of the adrenals of intact females and intact males of each reciprocal cross were compared and analyzed statistically (Tables 3 and 4). Average measurements of each pair of crosses were determined, and these were compared with all other pairs of crosses. The results of these computations are given in Tables 5–10.

From this statistical analysis it can be observed that the differences between pairs of crosses are greater and more significant than between reciprocals. The animals with the largest adrenals were YEL × CE, CE × C57BL and DBA × CE and their reciprocals, i.e., those with a CE parent.

Structure.—Changes observed in the adrenals of the intact animals can be divided into (*a*) those in the cortical elements and (*b*) those in the medullary area.

Subcapsular "A" cells noted in DBA × CE and CE × DBA F_1 females by 3 months (27) were present in all the other hybrid females at 2–4

TABLE 1

A. Body Weights of Intact F_1 Females over a Period of 1 Year

Age (months)	DBA/C57BL	C57BL/DBA	DBA/CE	CE/DBA	CE/C57BL	C57BL/CE	A/C3H	C3H/A	YEL/CE yel	Ag
6	24.0	26.0	31.0	28.0	25.0	26.0	22.0	27.0	35.0	26.0
7	26.0	25.0	29.0	30.0	24.0	28.0			33.0	33.0
8	29.0	22.0	34.0	28.0	29.0	32.0	27.0	31.0	32.0	26.0
9	27.0	28.0	29.0	31.0	27.0	30.0			50.0	26.0
10	30.0	27.0	30.0	30.0	28.0	28.0	28.0	30.0	33.0	30.0
11	23.0	28.0	32.0	34.0	32.0	26.0			41.0	26.0
12		26.0	32.0	46.0	34.0		29.0	28.0	53.0	26.0
13	25.0	28.0	30.0	28.0	37.0	29.0			37.0	29.0
14	25.0	24.0	33.0	34.0	31.0	31.0	33.0	34.0	40.0	30.0
15	25.0	22.0	32.0	28.0	32.0	30.0			44.0	29.0
16	26.0	23.0	29.0	27.0	28.0	32.0	42.0	31.0	38.0	33.0
17	28.0	27.0	28.0	28.0	29.0	31.0			38.0	22.0
18	27.0	26.0	30.0	29.0	33.0	28.0	36.0	36.0	51.0	28.0

B. Body Weights of Intact F_1 Males over a Period of 1 Year

Age (months)	DBA/C57BL	C57BL/DBA	DBA/CE	CE/DBA	CE/C57BL	C57BL/CE	A/C3H	C3H/A	YEL/CE yel	Ag
6	34.0	32.0	29.0	32.0	39.0	36.0	28.0	32.0	30.0	30.0
7	32.0	27.0	31.0	33.0	38.0	32.0			54.0	27.0
8	33.0	32.0	27.0	34.0	30.0	30.0	32.0	33.0	42.0	33.0
9	31.0	34.0	30.0	36.0	38.0	40.0			56.0	33.0
10	38.0	32.0	33.0	32.0	40.0	28.0	40.0	37.0	47.0	40.0
11	32.0	30.0	31.0	34.0	37.0	38.0			41.0	35.0
12	34.0	39.0	33.0	35.0	39.0	39.0		34.0	65.0	34.0
13	28.5	39.0	37.0	31.0	35.0	38.0			61.0	37.0
14	38.0	31.0	35.0	36.0	40.0	38.0	38.0	39.0	44.0	30.0
15	34.0	32.0	33.0	34.0	33.0	37.0			40.0	38.0
16	37.0		34.0	36.0	50.0	39.0	18.0	37.0	54.0	36.0
17	32.0	34.0	39.0	34.0	35.0	39.0			57.0	37.0
18	35.0	31.0	36.0	30.0	32.0	38.0	36.0	33.0		33.0

TABLE 2

A. Body Weights of Gonadectomized F_1 Females over a Period of 1 Year

Age (months)	DBA/C57BL	C57BL/DBA	DBA/CE	CE/DBA	CE/C57BL	C57BL/CE	A/C3H	C3H/A	YEL/CE yel	Ag
6	30.0				41.0	33.0	32.0	30.0	57.0	31.0
7	37.0	29.0	40.0	36.0	43.0	38.0			43.0	29.0
8	43.0	31.0	48.0	36.0	44.0	46.0	31.0	41.0	43.0	25.0
9	40.0	32.0	34.0	29.0	40.0	42.0			55.0	36.0
10	27.0	38.0	43.0	44.0	42.0	34.0	38.0	34.0	59.0	38.0
11	49.0	29.0	37.0	40.0	41.0	35.0			40.0	39.0
12	45.0	31.0	42.0	27.0	39.0	38.0	28.0	32.0	55.0	32.0
13	32.0	30.0	38.0	28.0	44.0	44.0			53.0	32.0
14	37.0	31.0	38.0	38.0	32.0	40.0	33.0	38.0	33.0	35.0
15	38.0	28.0	38.0	33.0	36.0	36.0			42.0	32.0
16	35.0	30.0	38.0	32.0	34.0	39.0	32.0	34.0	48.0	36.0
17	24.0	38.0	37.5	32.0	31.0	39.0				32.0
18	36.0	32.0	41.0	40.0	38.0	32.0	31.0	43.0	60.0	

B. Body Weights of Gonadectomized F_1 Males over a Period of 1 Year

Age (months)	DBA/C57BL	C57BL/DBA	DBA/CE	CE/DBA	CE/C57BL	C57BL/CE	A/C3H	C3H/A	YEL/CE yel	Ag
6	29.0		44.0		34.0	42.0	32.0	30.0	56.0	33.0
7	37.0	34.0	45.0	35.0	34.0	44.0			28.0	36.0
8	46.0	33.0	50.0	41.0		50.0	29.0	41.0	49.0	33.0
9	35.0	42.0	41.0	38.0	42.0	43.0			52.5	35.0
10	37.0	32.0	46.0	43.0	38.0	47.0	32.0	32.0	54.0	39.0
11	46.0	31.0	42.0	34.0	38.0	44.0			62.0	41.0
12	36.0	35.0	43.0	40.0	32.0	45.0	30.0	24.0	58.0	32.0
13	34.0	23.0	44.0	30.0	40.0	46.0			65.0	35.0
14	41.0	32.0	50.0	29.0	38.0	30.0	26.0	36.0	42.0	29.0
15	41.0	32.0	38.0	37.0	30.0	38.0			37.0	36.0
16	40.0	23.0	36.0	31.0	28.0	38.0	30.0	32.0	58.0	36.0
17	41.0	30.0	37.0	35.0	30.0	38.0			56.0	34.0
18	34.0	38.0	40.0	39.0	28.0	40.0	41.0	38.0	41.0	42.0

TABLE 3

STATISTICAL COMPARISON OF MEASUREMENTS RECORDED FOR RECIPROCAL GROUPS OF INTACT F_1 FEMALES

Cross	Total Adrenal	Cortex	Medulla	Ovary	Submaxillary	Thyroid
DBA×C57BL	$\bar{\Delta}$= .145	$\bar{\Delta}$= .109	=0	$\bar{\Delta}$= .847	$\bar{\Delta}$= .686	$\bar{\Delta}$= .595
and	t= .828	t= .641		t=1.104	t=6.660	t=1.667
C57BL×DBA	P> .4<.5	P> .5<.6		P> .2<.3	P> .01*	P> .1<.2
CE×C57BL	$\bar{\Delta}$= .176	$\bar{\Delta}$= .155	$\bar{\Delta}$= .0173	$\bar{\Delta}$= .128	$\bar{\Delta}$= .545	$\bar{\Delta}$= .392
and	t= .2567	t=1.107	t= .715	t= .2506	t= .2839	t=2.41
C57BL×CE	P> .8	P> .2<.3	P> .4<.5	P> .8<.9	P< .01†	P> .02<.05‡
A×C3H	$\bar{\Delta}$= .567	$\bar{\Delta}$= .55	$\bar{\Delta}$= .033	$\bar{\Delta}$= .566	$\bar{\Delta}$= .781	$\bar{\Delta}$= .178
and	t=1.327	t=1.403	t= .767	t= .552	t=2.56	t= .478
C3H×A	P> .2<.3	P> .1<.2	P> .4<.5	P> .5<.6	P< .02>.01§	P> .6<.7
YEL×CE (yel)	$\bar{\Delta}$= .31	$\bar{\Delta}$= .33	$\bar{\Delta}$= .03	$\bar{\Delta}$= .168	$\bar{\Delta}$= .200	$\bar{\Delta}$= .087
and	t=1.476	t=1.764	t=2.307	t= .126	t= .563	t= .388
YEL×CE (agouti)	P> .1<.2	P> .05<.1#	P> .02<.05#	P> .9	P> .5<.6	P> .7<.8

* C57BL×DBA larger.
† CE×C57BL larger.
‡ C57BL×CE larger.
§ C3H×A larger.
# Agouti larger.

TABLE 4

STATISTICAL COMPARISON OF MEASUREMENTS RECORDED FOR RECIPROCAL GROUPS OF INTACT F_1 MALES

Cross	Total adrenal	Cortex	Medulla	Submaxillary	Thyroid
DBA×C57BL	$\bar{\Delta}$= .208	$\bar{\Delta}$= .179	$\bar{\Delta}$= .044	$\bar{\Delta}$= .142	$\bar{\Delta}$= .02
and	t= .156	t= .124	t= .041	t= .500	t= .054
C57BL×DBA	P> .1<.2	P> .1<.2	P> .2<.3	P> .6<.7	P>1
CE×C57BL	$\bar{\Delta}$= .200	$\bar{\Delta}$= .059	$\bar{\Delta}$= .111	$\bar{\Delta}$=1.064	$\bar{\Delta}$= .517
and	t=1.183	t= .468	t=1.651	t=3.841	t=3.293
C57BL×CE	P> .2<.3	P> .6<.7	P> .1<.2	P< .01*	P< .01†
A×C3H	$\bar{\Delta}$= .384	$\bar{\Delta}$= .322	$\bar{\Delta}$= .007	$\bar{\Delta}$= .343	$\bar{\Delta}$= .928
and	t=1.7	t=1.894	t= .205	t= .513	t=3.022
C3H×A	P> .1<.2	P> .05<.1	P>.8<.9	P> .6<.7	P< .01‡
YEL×CE (yel)	$\bar{\Delta}$= .121	$\bar{\Delta}$= .226	$\bar{\Delta}$= .121	$\bar{\Delta}$= .135	$\bar{\Delta}$=1.30
and	t= .62	t=1 .559	t=2.574	t= .374	t=2.584
YEL×CE (ag)	P> .4<.5	P> .1<.2	P> .01<.02§	P> .7<.8	P> .01<.02§

* C57BL×CE larger.
† CE×C57BL larger.
‡ A×C3H larger.
§ Agouti larger.

TABLE 5

STATISTICAL COMPARISON OF THE AVERAGE OF TOTAL ADRENAL MEASUREMENTS FOR VARIOUS F_1 INTACT FEMALES

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE	$\bar{\Delta}$= .695			
and	t=4.081			
CE×DBA	P< .01*			
CE×C57BL	$\bar{\Delta}$=1.077	$\bar{\Delta}$= .476		
and	t=5.494	t=3.152		
C57BL×CE	P< .01†	P< .01†		
A×C3H	$\bar{\Delta}$= .09	$\bar{\Delta}$= .555	$\bar{\Delta}$=1.033	
and	t= .246	t=1.154	t=2.784	
C3H×A	P> .8<.9	P> .2<.3	P> .01<.02†	
YEL×CE (yel)	$\bar{\Delta}$=1.113	$\bar{\Delta}$= .300	$\bar{\Delta}$= .035	$\bar{\Delta}$=1.034
and	t=8.306	t=1.220	t= .232	t=3.241
YEL×CE (ag)	P< .01‡	P> .2<.3	P> .8<.9	P> .01<.02‡

* DBA×CE F_1 and reciprocal larger.
† CE×C57BL F_1 and reciprocal larger.
‡ YEL×CE larger.

TABLE 6

Statistical Comparison of the Averages of Adrenal Cortical Measurements for Various F_1 Intact Females

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$= .561 t=3.281 P< .01*			
CE×C57BL and C57BL×CE	$\bar{\Delta}$=1.045 t=1.219 P> .2<.3	$\bar{\Delta}$= .392 t=2.820 P< .01†		
A×C3H and C3H×A	$\bar{\Delta}$= .10 t= .291 P> .7<.8	$\bar{\Delta}$= .427 t= .964 P> .3<.4	$\bar{\Delta}$= .834 t=2.310 P> .02<.05†	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$= .773 t=6.135 P< .01‡	$\bar{\Delta}$= .300 t=1.253 P> .2<.3	$\bar{\Delta}$= .045 t= .298 P> .7<.8	$\bar{\Delta}$= .723 t=2.639 P> .02<.05‡

* DBA×CE and reciprocal larger.
† CE×C57BL and reciprocal larger.
‡ YEL×CE larger.

TABLE 7

Statistical Comparison of the Averages of Medullary Measurements for Various F_1 Intact Females

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$= .128 t=6.400 P< .01*			
CE×C57BL and C57BL×CE	$\bar{\Delta}$= .231 t=7.700 P< .01†	$\bar{\Delta}$= .066 t=3.474 P< .01†		
A×C3H and C3H×A	$\bar{\Delta}$= .03 t= .600 P> .5<.6	$\bar{\Delta}$= .127 t=1.814 P< .1	$\bar{\Delta}$= .2 t=3.225 P< .01†	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$= .326 t=9.056 P< .01‡	$\bar{\Delta}$= .147 t=4.742 P< .01‡	$\bar{\Delta}$= .085 t=5.00 P< .01‡	$\bar{\Delta}$= .3 t=3.464 P< .01‡

* DBA×CE and reciprocal larger.
† CE×C57BL and reciprocal larger.
‡ YEL×CE larger.

TABLE 8

Statistical Comparison of the Averages of Total Adrenal Measurements for Various F_1 Intact Males

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$= .191 t=2.729 P> .01<.02*			
CE×C57BL and C57BL×CE	$\bar{\Delta}$= .682 t=3.461 P< .01†	$\bar{\Delta}$= .400 t=3.372 P< .01†		
A×C3H and C3H×A	$\bar{\Delta}$= .373 t=1.444 P> .1<.2	$\bar{\Delta}$= .533 t=2.896 P> .01<.02*	$\bar{\Delta}$=1.032 t=3.297 P< .01†	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$= .663 t=5.715 P< .01‡	$\bar{\Delta}$= .451 t=4.421 P< .01‡	$\bar{\Delta}$= .168 t=1.070 P> .2<.3	$\bar{\Delta}$= .934 t=4.043 P< .01‡

* DBA×CE and reciprocal larger.
† CE×C57BL and reciprocal larger.
‡ YEL×CE larger.

months of age. In older animals these "A" cells formed heavy bands but seldom formed nodules. The "A" cells were present in at least one adrenal of all females of all crosses up to the oldest animal studied. Occasionally, tumors presumably arising from these cells were found. There were two in CE × DBA F_1 females and one in a 22-month C3H × AF_1 female. The bands of "A" cells were not so heavy in the adrenals of the yellow and non-yellow F_1 females as they were in the adrenals of the rest of the hybrid females.

Fasciculata nodules such as those described for a CE × DBA F_1 female at 22 months of age (27) were not found in any of the other hybrid females. There were no other striking changes in the zona fasciculata of any of the hybrid females.

The changes noted in the inner zone of the cortex make the groups of females more distinct from one another than the changes in the zona glomerulosa and the zona fasciculata. Several alterations were observed in this zone. First, the x-zone disappeared in all the F_1 females when they were 5–8 months of age. Second, unlike the disturbance of the x-zone under "A" cells of gonadectomized animals, there was a characteristic and different change under the bands of the "A" cells of the intact females. There were small clusters of dark cells and irregular spaces, similar to but not blood sinuses, under the "A" cells. Third, in the absence of any other alterations in the cortex in old animals over a year of age the inner zone appeared to be stretched, lacy, and pulled apart. This variation was similar to the appearance of the degeneration of the x-zone area under A cells of gonadectomized animals and may possibly be related to x-zone degeneration. Fourth, most striking was the appear-

TABLE 9

STATISTICAL COMPARISON OF THE AVERAGES OF ADRENAL CORTICAL MEASUREMENTS FOR VARIOUS F_1 INTACT MALES

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$ = .053 t = .089 P> .9			
CE×C57BL and C57BL×CE	$\bar{\Delta}$ = .376 t=3.615 P< .01*	$\bar{\Delta}$ = .267 t=3.338 P< .01*		
A×C3H and C3H×A	$\bar{\Delta}$ = .290 t=1.45 P> .1<.2	$\bar{\Delta}$ = .283 t=1.862 P> .05<.1	$\bar{\Delta}$ = .658 t=3.074 P< .01*	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$ = .375 t=4.076 P< .01†	$\bar{\Delta}$ = .331 t=4.299 P< .01†	$\bar{\Delta}$ = .116 t=1.137 P> .2<.3	$\bar{\Delta}$ = .622 t=3.380 P< .01†

* CE×C57BL and reciprocal larger.
† YEL×CE larger.

TABLE 10

STATISTICAL COMPARISON OF THE AVERAGES OF MEDULLARY MEASUREMENTS FOR VARIOUS F_1 INTACT MALES

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$ = .152 t=1.187 P> .2<.3			
CE×C57BL and C57BL×CE	$\bar{\Delta}$ = .338 t=5.365 P< .01*	$\bar{\Delta}$ = .128 t=2.415 P> .02<.05*		
A×C3H and C3H×A	$\bar{\Delta}$ = .073 t=1.263 P> .2<.3	$\bar{\Delta}$ = .2166 t=3.807 P< .01†	$\bar{\Delta}$ = .383 t=3.683 P< .01*	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$ = .306 t=9.27 P< .01‡	$\bar{\Delta}$ = .077 t= .181 P> .8<.9	$\bar{\Delta}$ = .0632 t=1.400 P> .1<.2	$\bar{\Delta}$ = .311 t=6.91 P< .01‡

* CE×C57BL and reciprocal larger.
† DBA×CE and reciprocal larger.
‡ YEL×CE larger.

ance of cells containing lipochrome in the inner or juxta-medullary zone. In DBA × C57BL F_1 females it was found occasionally after 22 months; in C57BL × DBA F_1 females after 16 months, it was noted sporadically. In CE × C57BL F_1 females it first appeared at 11 months and in the reciprocal C57BL × CE F_1 females it was first found at 7 months. The lipochrome cells were then a constant feature of the adrenals of CE × C57BL and C57BL × CE F_1 females up to the oldest ages studied. Not only was it abundant in the juxta-medullary zone, but there were clusters in the cortex and scattered through the medulla. Lipochrome cells were present in the yellow females after 8 months and after 15 months in agouti females. The lipochrome cells were constant in these series but were limited to the juxta-medullary zone.

Fifth, hyalinization occurred, originating perhaps in the lower portion of the zona fasciculata and in the juxta-medullary zone, and was found in the adrenals of several of the groups of intact females. The hyaline was homogeneous, lacked cellular elements, and in extreme cases appeared to fill the entire area between the medulla and the zona glomerulosa. DBA × CE, CE × DBA, and C57BL × CE F_1 intact female adrenals frequently had hyaline changes. This was occasionally found in C57BL × DBA, CE × C57BL, and A × C3H F_1 female adrenals but was not observed in the other hybrid groups.

Medullary alterations were infrequent. As previously mentioned, cells containing lipochrome were found within the medulla in CE × C57BL and C57BL × CE females. The other change observed was the appearance of small nodules. These nodules were more cellular than normal medullary tissue, no sinusoids were evident, the nodules were surrounded by a thin layer of connective tissue, and mitotic figures were present in the nodules. Special staining of these nodules was not possible; therefore, further detailed study of them could not be made. However, it may be permissible to call these nodules medullary adenomas. In addition to the one adenoma found in a 20-month CE × DBA female (27), adenomas occurred at 21 and 23 months in DBA × C57BL females, at 16 and 25 months in CE × C57BL females, at 29 months in C57BL × CE females, and at 28 and 30 months in C3H × A females.

Some of the changes observed in the adrenals of intact males were similar to those described in the females, but there were a few striking differences. The "A" cells appeared from 3 to 6 months of age, a little later than in the females. There were changes similar to those found in females in the juxta-medullary zone under these "A" cells. Bands of "A" cells were not so extensive as those in females, and there were no "A" cell tumors in any of the hybrid males. The yellow and nonyellow males seemed to have fewer "A" cells than the males in the other hybrid series.

Cells somewhat similar to the enlarged "B"[2] cells were observed within the bands of "A" cells in a few of the adrenals of the YEL × CE series.

Fasciculata nodules, which had been observed in CE × DBA females, (27), were found much more frequently in the hybrid males. There were disturbances of the orderly pattern of the zona fasciculata in DBA × C57BL, C57BL × DBA, and C3H × A males, but no distinct nodules. Distinct fasciculata nodules were noted in several of the males of the DBA × CE, CE × DBA, CE × C57BL, C57BL × CE, and A × C3H series. No disturbances were noted in the YEL × CE series.

TABLE 11

STATISTICAL COMPARISON OF MEASUREMENTS RECORDED FOR RECIPROCAL GROUPS OF GONADECTOMIZED MALES AND FEMALES

Cross	Total adrenal	
DBA×C57BL	$\bar{\Delta}$= .068	$\bar{\Delta}$= .726
and	t= .228	t= .957
C57BL×DBA	P> .8< .9	P> .3< .4
CE×C57BL	$\bar{\Delta}$=51.16	$\bar{\Delta}$=715.9
and	t= 1.252	t= 1.521
C57BL×CE	P> .2< .3	P> .1< .2
A×C3H	$\bar{\Delta}$=18.422	$\bar{\Delta}$= .242
and	t= 1.269	t= .466
C3H×A	P> .2< .3	P> .6< .7
YEL×CE	$\bar{\Delta}$=53.94	$\bar{\Delta}$= 10.300
and	t= .207	t= .0295
CE×YEL	P> .8< .9	P> .9

Alterations in the juxta-medullary zone were similar to those found in the females. Lipochrome-containing cells were found in only one DBA × C57BL male at 24 months, while none were present in C57BL × DBA, A × C3H, and C3H × A males. CE × C57BL and C57BL × CE males had increasingly large numbers of lipochrome cells from 6 months of age on, and, as in the females, this substance was found in the cortex and within the medulla. YEL × CE yellow males had lipochrome cells after 13 months. Lipochrome cells were observed in nonyellows after 9 months, and the cells were limited to the juxta-medullary zone. Hyaline change was observed more frequently and was more extreme in the hybrid males. Hyaline was found in DBA × C57BL, C57BL × DBA, DBA × CE, CE × DBA, CE × C57BL, and

[2] "B" cells, when they occur in the adrenals of gonadectomized animals, are thought to be hypertrophied cells of the zona fasciculata.

C57BL × CE males. None of the other groups underwent this hyaline change.

Only one medullary adenoma was found in all the hybrid male series. It was morphologically similar to those observed in the females. This adenoma occurred in a 26-month A × C3H male.

Gonadectomized Animals

The first and most outstanding feature of the gonadectomized series of hybrid animals was that adrenal cortical carcinomas occurred in all the crosses.

The response of the DBA × CE and CE × DBA females and males to gonadectomy has been reported (27). It is relevant, however, to review certain of these findings at this time. The changes leading to the development of adrenal cortical carcinoma occurred in the same stages as those observed in strain CE (9, 31, 33). "A" cells appeared at 2 months of age, "B" cells about 4 months of age, and cortical neoplasms at 6 months in CE × DBA gonadectomized females and at 9 months in DBA × CE gonadectomized females. Adrenal cortical carcinomas appeared in both DBA × CE and CE × DBA castrate males at 12 months of age. Morphologically, the tumors were similar to those of strain CE, and the same variations in tumor structure were observed. The tumors grew to very large size and killed all DBA × CE females before 22 months, the DBA × CE males around 24 months, CE × DBA females at about 24 months, and CE × DBA males at about 26 months of age.

The response of the adrenals of the females of the other hybrid groups to gonadectomy varies distinctly from the response of the DBA × CE and CE × DBA gonadectomized females. In DBA × C57BL and C57BL × DBA gonadectomized females the early stages of change in the adrenals were similar to DBA × CE and CE × DBA. "A" cells were present at 3 months, and "B" cells were noted from 4 months on. The adrenals had nodular hyperplasia for many months. At 14 months in DBA × C57BL and at 15 months in C57BL × DBA changes similar to true adrenal cortical carcinomas were observed. Such carcinomas were found frequently, but not in 100 per cent, throughout the later ages. The tumors were small; none were large enough to measure macroscopically, and they were cystic in many instances. The structure was similar to the ovary. Tumors were first noted at 10 months of age in both CE × C57BL and C57BL × CE gonadectomized females. The tumors occurred sporadically in the CE × C57BL group until at 15 months, and then they appeared in 100 per cent of the animals in the rest of the series up to 26 months of age. C57BL × CE gonadectomized females had 100 per cent adrenal cortical carcinoma occurrence from 10 months through 28 months of age. There was more size variation in tumors in these series than in those of DBA × CE and CE × DBA gonadectomized females. However, the largest tumor ever observed either in strain CE or in these hybrids was in a CE × C57BL gonadectomized female; the tumor was 19,000 c. mm. in size.

Adrenal cortical carcinomas occurred in all A × C3H gonadectomized females from 16 through 24 months of age and in all C3H × A gonadectomized females from 10 through 30 months of age. With the exception of one adrenal tumor in C3H × A that was grossly measurable, all the tumors in the A × C3H and C3H × A series were of microscopic size.

YEL × CE gonadectomized females had adrenal tumors at 13 months of age. They were more consistently present and larger in the nonyellow gonadectomized females than in the yellow gonadectomized females.

Castrate hybrid males in general had fewer adrenal cortical tumors than gonadectomized females. However, the general type of response of the groups followed the pattern of the females. There were only two tumors in DBA × C57BL castrate males (at 16 and 19 months of age). All other males had extensive nodular hyperplasia. Three tumors (at 15, 18, and 20 months) were observed in the C57BL × DBA castrate male series. These five tumors were all microscopic in size. The first adrenal tumor in the CE × C57BL male group was found at 16 months of age, and tumors occurred sporadically in the rest of the series. However, in C57BL × CE castrate males, the first tumor was present at 10 months of age, and there was 100 per cent occurrence of the tumors after 16 months.

Adrenal tumors occurred in both A × C3H and C3H × A castrate males at 16 months and were present in 100 per cent of the animals through 28 and 32 months of age, respectively. Three of the tumors in these series were grossly recognizable, the rest were all microscopic. Yellow F_1 males had adrenal tumors at 10 months, while in the nonyellow F_1 males tumors occurred at 17 months of age. These tumors were variable in size.

Morphologically, the tumors in all the hybrid groups whether large or small were comparable to those of strain CE and had the same variations in structure that have previously been described (9, 31, 33–35).

All the adrenals of the gonadectomized animals were measured. Total adrenal size and cortical and

medullary size were calculated. Cortical and medullary size were not determined, however, when the adrenals became abnormal. Significance of the mean difference of the measurements of the reciprocal groups only was computed (Table 11). The results of the computations divided the hybrids into three groups. In the first group, DBA × CE and CE × DBA series, there were large adrenal tumors. The second group, CE × C57BL, C57BL × CE, and YEL × CE showed very variable tumor size. The third group, DBA × C57BL, C57BL × DBA, A × C3H, and C3H × A, had small tumors.

reciprocal groups were compared as above. Again, however, numbers of cells measured were too few to establish real significance.

In gonadectomized hybrid females and males first evidence of stimulation from the abnormal adrenal cortices was found in the terminal tubule cells of the submaxillary gland. Cell heights varied from animal to animal, but they rose in many instances to an intermediate height of 17–19 μ, and in many instances where there were also adrenal cortical carcinomas the cell heights were the same as that of normal males. The nuclei, however, were not quite so uniformly basal as in normal males.

TABLE 12

STATISTICAL COMPARISON OF THE AVERAGES OF OVARIAN MEASUREMENTS FOR VARIOUS F_1 INTACT FEMALES

	DBA×C57BL and C57BL×DBA	DBA×CE and CE×DBA	CE×C57BL and C57BL×CE	A×C3H and C3H×A
DBA×CE and CE×DBA	$\bar{\Delta}$=14.753 t= 5.180 P< .01*			
CE×C57BL and C57BL×CE	$\bar{\Delta}$= 1.166 t= 2.546 P> .02<.05†	$\bar{\Delta}$= 9.48 t= 3.953 P< .01*		
A×C3H and C3H×A	$\bar{\Delta}$= .430 t= .441 P> .6<.7	$\bar{\Delta}$=13.518 t= 3.299 P< .01*	$\bar{\Delta}$=1.417 t=2.218 P> .02<.05†	
YEL×CE (yel) and YEL×CE (ag)	$\bar{\Delta}$= 7.224 t= 5.460 P< .01‡	$\bar{\Delta}$= 3.132 t= 1.166 P> .2<.3	$\bar{\Delta}$=4.710 t=4.687 P< .01‡	$\bar{\Delta}$=6.178 t=4.489 P< .01‡

* DBA×CE and reciprocal larger.
† CE×C57BL and reciprocal larger.
‡ YEL×CE larger.

OTHER ORGANS

Submaxillary.—The cells of the terminal tubules of the submaxillary gland in mice have been studied and found to be grossly and histologically different in males and females (7). The terminal tubule cells have been observed histologically and seem to be extremely sensitive to the presence of androgens. The terminal tubule cell heights were used to determine androgenic activity in these series of hybrid animals. The cell heights in the intact females were usually between 12 and 16 μ, and the nuclei were centrally located. The significance of the difference between means of the various reciprocal groups was computed (Tables 3 and 4). However, since relatively few cells were measured to obtain an average, it was felt that real differences were not demonstrated. DBA × CE, CE × DBA, and both groups of YEL × CE females had an average cell height of between 14 and 17 μ. No other differences were noted in the intact hybrid females.

The cell height in the terminal tubules of intact hybrid males was between 18 and 26 μ, and nuclei were very basal. The average cell heights of the re-

The reciprocal group measurements were not analyzed statistically.

Thyroid.—An attempt was made to find a measure of thyroid activity by determining the heights of the follicular cells. The cell height measurements of the reciprocal groups were analyzed statistically (Tables 3 and 4). Some groups had statistically significant differences, but, as with the submaxillary gland, it was felt that the number of cells measured was too small to be strikingly significant. There was too much variation from animal to animal.

In the intact and gonadectomized females and males there were no unusual structural changes. Stromal material between the follicles increased in amount in all groups in older animals.

Ovary.—The left ovaries of all intact females were measured in the same manner as the adrenals. The sizes of the ovaries of the reciprocal groups were compared statistically, as were the measurements of the paired hybrid groups (Table 12). There were tremendous size differences between hybrid groups. Largest ovaries were found in the DBA × CE and CE × DBA female

groups, with YEL × CE females showing the next largest. CE × C57BL, C57BL × CE ovaries were moderate-sized, the DBA × C57BL and C57BL × DBA ovaries were smaller, and the A × C3H and the C3H × A ovaries were the smallest.

There were distinct differences in the hybrid groups in the amount of lipochrome cells, in the retention and hyalinization of corpora lutea, in the occurrence of cysts, and in the appearance of granulosa-cell tumors.

The number of lipochrome cells increased steadily in amount from 4 to 6 months in CE × C57BL and C57BL × CE females, while DBA × C57BL, C57BL × DBA, and YEL × CE series of females also had consistent numbers of lipochrome cells in the ovaries. Hyalinization and calcification of the corpora lutea were extreme in DBA × CE and CE × DBA females, and there was some hyalinization in the DBA × C57BL and C57BL × DBA series. There was beginning hyalinization in the centers of a few corpora lutea of CE × C57BL, C57BL × CE, A × C3H, and C3H × A females. Cysts occurred frequently in the DBA × C57BL and C57BL × DBA ovaries, and there were a few noted in A × C3H and C3H × A ovaries.

One papilliferous growth in a cyst was found in a 14-month C57BL × DBA ovary, and a few granulosa-cell tumors were noted in the old DBA × CE and CE × DBA females.

Testis.—Testicular structure varied little from that described for DBA × CE and CE × DBA intact males (27). Spermatogenesis was beginning at 1 month, had increased by 2 months, and was reduced after 20–23 months. Testes of CE × C57BL and C57BL × CE males had some lipochrome cell deposition in the Leydig cells of the stroma. No abnormalities were noted.

Vesicular glands and prostates.—There were no noticeable changes or differences in these glands in any of the intact hybrid males except that after 24 months there was not so much secretion in the vesicular glands. In gonadectomized males of all hybrid groups there was stimulation of these accessory reproductive organs. Stimulation was noted first between 6 and 12 months in the various crosses, depending upon the amount of alteration occurring in the adrenal cortices. There was evidence of estrogenic and androgenic types of stimulation, i.e., squamous metaplasia of the prostatic epithelium, hyalinization of the stroma, and presence of secretion in the vesicular glands. The vesicular glands and prostates of none of the hybrid groups had exclusively feminizing or masculinizing changes. The situation varied from animal to animal. The glands in the DBA × CE and CE × DBA castrated males more nearly attained the size found in intact males than did any of the other groups.

Uterus.—Uterine changes in intact and gonadectomized DBA × CE and CE × DBA females have been discussed previously (2). The major changes were: cystic glandular hyperplasia, which occurred at 8 months in both control and gonadectomized DBA × CE and CE × DBA females; adenomyosis, which was apparent at 7 months in CE × DBA females, at 9 months in DBA × CE females; and at 14 months in the gonadectomized groups; and adenomatous hyperplasia, which appeared about 11–12 months in the intact females and 16 months or later in the gonadectomized females.

Observation of the other intact hybrid females, except YEL × CE F_1 females, revealed that none had such extreme alterations in their uteri. There was some mild cystic glandular hyperplasia, especially in CE × C57BL and C57BL × CE females, and a few large cysts. Adenomyosis and adenomatous hyperplasia were not observed. In A × C3H and C3H × A females there was little cystic glandular hyperplasia, but the lumen was frequently distended. The uteri of the YEL × CE females had changes more similar to those of the DBA × CE and CE × DBA females, but the uteri did not become so large or so abnormal as in the DBA × CE and CE × DBA females.

The gonadectomized hybrid females, except DBA × CE and CE × DBA, showed evidence of uterine stimulation at about the same time the vaginal epithelium began to change. In the uteri in DBA × C57BL, C57BL × DBA, and A × C3H gonadectomized females there was very little cystic glandular hyperplasia. This hyperplasia was noticed in a few C3H × A, in some CE × C57BL and C57BL × CE, and in many YEL × CE gonadectomized females.

Vagina.—Since intact females were sacrificed at different time intervals, regardless of the stage of the estrous cycle, the condition of the vaginal epithelium varied greatly at the time of autopsy. All crosses showed that there was evidence of change from diestrus at 1–2 months of age. The evidence of estrous cycle continued until the animals were aged. Beyond 24 months of age the vaginas showed evidence of either a weak phase of the cycle or a diestrous picture. No particular differences were noted in the reciprocal crosses.

The females that were gonadectomized presented very clear evidence of hormonal stimulation, presumably derived from the activity of the abnormal adrenals. It has been shown that DBA × CE and CE × DBA mice first had a change in the vaginal epithelium at 7 and 6 months of age, re-

spectively, and thereafter the vaginal epithelium was most usually at metestrous phase until old age. This held true for A × C3H and C3H × A gonadectomized females which had first evidence of cyclic activity at 12 and 8 months, respectively, and continued in metestrus until old age. There was much more variation in the other hybrid groups.

The time of first indications of stimulation of the vagina varied greatly. In DBA × C57BL animals it occurred at 8 months, while in the C57BL × DBA series no change was noted until 11 months. Among the CE × C57BL gonadectomized females, a change from diestrus was not observed until 12 months, but in C57BL × CE animals the first change occurred at 9 months. In the YEL × CE series vaginal stimulation was noted in the gonadectomized yellow animals at 10 months and at 13 months in the nonyellow gonadectomized females.

The stages of the estrous cycles of these gonadectomized females at autopsy were more comparable to the random assortment found in intact females, than to the metestrous condition noted in the DBA × CE, CE × DBA, A × C3H, and C3H × A series.

No outstanding abnormalities such as precancerous lesions or tumors of the vagina were found in either the intact or gonadectomized females of any of the hybrid groups.

Pituitary.—Details of the histology of the hypophyses of the normal and gonadectomized animals of this entire series of crosses with the exception of the YEL × CE series have been fully discussed in a previous report (3, 5). Therefore, it need only be recalled that several changes were noted in the hypophyses.

Many of the hypophyses of intact animals contained cysts lined by ciliated epithelium or cysts lined with cuboidal epithelium. There was a statistically significant difference between control females and males in the occurrence of nonciliated cysts. There was no significant difference, however, when comparing all castrate animals with all intact animals. Only one chromophobe adenoma was found among all intact animals examined, and that was in a 30-month-old DBA × C57BL female.

Some basophile adenomas occurred in all the gonadectomized series of the F_1 hybrids but were most numerous in DBA × CE and CE × DBA gonadectomized animals and were frequent in the CE × C57BL and C57BL × CE gonadectomized groups. Several occurred in the A × C3H experimental group and very few in all the other series.

Mammary glands.—The mammary glands of the intact females were not unusual. A few mammary tumors were found in the DBA × C57BL and DBA × CE intact females, many in A × C3H and C3H × A females. The latter two reciprocals were distinctly different. Among the A × C3H females the mammary tumors appeared earlier, and none of the mice lived beyond 24 months of age, while in the C3H × A series the tumors occurred later and some females attained 32 months of age.

In intact males the mammary glands were rudimentary. However, the C57BL × DBA and C57BL × CE male series had larger gland rudiments than their reciprocals, the DBA × C57BL and CE × C57BL males.

The mammary glands of both the gonadectomized females and males responded in a similar manner; when the adrenals became abnormal, either with nodular hyperplasia or cortical carcinoma, there was growth of the mammary glands. The epithelium of the mammary glands of those animals with large adrenal tumors was similar to that found in virgin females. If basophile adenomas of the anterior pituitary were present, in addition to the adrenal tumors, the epithelium showed secretory vacuoles, and the alveoli and ducts were filled with secretion. In the DBA × CE series, where the MTI was present, and in those animals in which both adrenal and pituitary tumors occurred, mammary tumors did appear in both gonadectomized females and males. This was not always true in the A × C3H and C3H × A series.

Body weight.—Body weights of the various intact and gonadectomized groups have not been analyzed statistically. A separate study was made of weight comparisons of intact yellow and nonyellow females and males (4). The intact and gonadectomized yellow groups were heavier than the nonyellow groups. In the rest of the hybrid series, at least up to 10–12 months of age, the gonadectomized animals were heavier than the intact animals. In general, they followed the pattern of the DBA × CE and CE × DBA series (27); the intact females were the lightest, the gonadectomized males the heaviest (Tables 1 and 2).

TUMORS OF VARIOUS SITES

Tumors other than those mentioned previously (adrenal, pituitary, mammary, ovarian) were observed. They included primary papillary lung adenomas, lymphoblastomas, sarcomas, papillomas, hepatomas, hemangiomas, and reticuloendotheliomas. Not enough animals of the various crosses were examined so that any indication of incidence of these tumors could be given.

DISCUSSION

A pilot experiment such as this does not permit extensive discussion, but various factors can be emphasized, and certain speculations may be permitted.

The outstanding features of this series of hybrid crosses are (*a*) the aging changes in the adrenals of the intact animals, (*b*) the differences in ovarian size and structure, (*c*) adrenal cortical tumor occurrence in gonadectomized animals, (*d*) uterine changes in intact and gonadectomized females, (*e*) appearance of basophile tumors in the anterior pituitary of gonadectomized animals, and (*f*) mammary gland changes and tumor incidence in intact and gonadectomized animals.

The hybrid crosses were set up so that two specific things might be investigated—chromosomal inheritance and parental influence of any other type. Therefore, let us consider first those results which at present seem to be attributable to parental influence without clear genic dependence.

The *time* at which various changes occur seems to vary between the reciprocals more than the size and morphological changes of the various glands. Most of these changes are not consistently uniform from cross to cross. Two striking examples of this are the time of appearance of adrenal cortical tumors and the time of first evidence of stimulation of the vaginal epithelium of gonadectomized females.

Adrenal tumors were first observed at 9 months in DBA × CE and at 6 months in CE × DBA gonadectomized females, while they were first noted at 12 months in both DBA × CE and CE × DBA castrate males. CE × DBA animals have CE mothers. Another pair of crosses of this type are CE × C57BL and C57BL × CE. Adrenal tumors first appeared in both CE × C57BL and C57BL × CE gonadectomized females at 10 months. CE × C57BL castrate males did not have adrenal tumors until 16 months, and then their appearance was sporadic, while C57BL × CE castrate males had them at 10 months, and there was a 100 per cent occurrence in all C57BL × CE castrate males after 16 months. CE × C57BL animals have CE mothers. Animals of the YEL × CE series cannot be compared, since no reciprocal cross was made.

The variation in the time of stimulation of the vaginal epithelium of gonadectomized females followed a confusing pattern. There was a 3 months' difference between DBA × C57BL and C57BL × DBA, and CE × C57BL and C57BL × CE, and only 1 month difference between DBA × CE and CE × DBA. DBA × C57BL, C57BL × CE, and CE × DBA females were stimulated earlier than the reciprocals although they do not have the same strain mothers.

Pituitary tumors occurred more frequently in CE × DBA and C57BL × CE gonadectomized animals. One had CE mothers and the other C57BL mothers.

Not all evidences of parental influence were so inconsistent. Mammary tumor occurrence in DBA × C57BL and DBA × CE females was expected and observed; both had DBA mothers. However, mammary tumor occurrence in the A × C3H and C3H × A crosses was not what might have been expected. The A × C3H virgin females had tumors earlier and more frequently than did C3H × A. The A × C3H females had A/Wy mothers. A virgin females have an extremely low mammary tumor incidence, while virgin C3H females have as high an incidence as C3H breeding females. Other workers have had varying results with crosses of these strains. Andervont and Heston (15) reported no reciprocal differences, while Bittner (1) and his associates had similar results to these reported here. It is possible that this variation may be one instance where subline differences of inbred strains may be operative. Strains A/Wy and C3H are descendants of Bittner's strains, while the strains used by Andervont and Heston have somewhat different ancestries.

The variations in time of occurrence of the above-mentioned changes indicate that parental influence does occur but might be modified by the genetic combinations employed.

There are several characteristics observed in the hybrids that have been inherited, without evidence of any parental influence other than of a genic nature. First, as shown in DBA × CE and CE × DBA gonadectomized animals, the response to gonadectomy is of the same type as that found in strain CE and not in strain DBA. That is, the occurrence of adrenal cortical carcinoma is dominant or epistatic to the change to nodular hyperplasia only. Also, from the observed response of CE × C57BL and C57BL × CE animals, it is evident that the carcinoma change is dominant to lack of any change such as that observed in strain C57BL/10.

However, this does not explain the response to gonadectomy that was observed in the other hybrid cross of DBA and C57BL and A and C3H. These animals also had adrenal cortical tumors, even though there was no such response in any of their parental strains. It has been shown in other experiments (20), and it is quite reasonable to assume, that in these series hybridization itself can produce additive or new changes in internal imbalance that have not been found in the parental strains. It is interesting to note here that all the

adrenal tumors in these series were quite small, i.e., they actually produced the tumors, but perhaps they had an inhibiting factor or lacked a growth factor that prevented the tumors from becoming as large as those found in strain CE and its hybrids. Another comparison that properly enters this discussion is that adrenal size in intact animals divides the hybrids in the same groups; those with a CE parent have larger adrenals than those that have no inheritance from strain CE.

Hybridization also seems to have produced cumulative or stimulative effects on structural changes in the uteri. Strains CE and DBA both have the alterations noted, but the alterations are not so extreme as those observed in the DBA × CE and CE × DBA animals (2). When these two strains (CE and DBA) were crossed with strain C57BL/10, the changes were repressed in the DBA × C57BL, C57BL × DBA, CE × C57BL, and C57BL × CE females.

Characteristic variations of ovarian structure and size are inherited. The nodules of lipochrome cells, which were found in strain C57BL (8), occur in C57BL hybrids DBA × C57BL, C57BL × DBA, CE × C57BL, and C57BL × CE. The size of the ovary is reduced in DBA × C57BL and C57BL × DBA and comparable in DBA × CE and CE × DBA to the size of strain DBA ovaries. Hyalinization of the corpora lutea and cyst formation, another feature of DBA ovaries, was noted in the hybrids of DBA—DBA × C57BL, C57BL × DBA, DBA × CE, and CE × DBA. Granulosa-cell tumors, found in old CE females, were found occasionally in DBA × CE and CE × DBA females but not in other CE hybrids, CE × C57BL, C57BL × CE, or in YEL × CE yellow and nonyellow females.

In the YEL × CE series there was an opportunity to study the effect of the presence or absence of a single gene—the yellow gene (A^y). The size of adrenals and ovaries were not affected by this gene. The time of the appearance of "A" cells in the outer layer of the adrenal cortex differed. In yellow (A^y) females and males, "A" cells were noticeable at 2 months of age. They were not found in nonyellow (A) females until 4 months of age and in nonyellow (A) males until 8 months. Lipochrome-containing cells in the adrenal and ovary occurred earlier in yellow animals. Adrenal cortical tumors occurred at the same time in both groups (A^y and A) of gonadectomized females. They were, however, observed 7 months earlier in yellow castrate males than in nonyellow castrate males. The tumors were larger in the yellow groups but were more consistently present in the nonyellow groups.

There has been extensive speculation as to the mechanisms that are involved in causing the structural changes in the adrenal, pituitary, and uterus, particularly in the DBA × CE and CE × DBA animals (2, 3, 5).

Our present knowledge on the biogenesis of adrenal cortical steroid hormones and their derivatives invites speculation on the possible bearing that this type of experimentation could have on determining the structure and function of the adrenals of the F_1 animals and of their parental inbred strains. There is in the hybrids, in spite of the fact that they all developed adrenal cortical carcinomas, a differential response in the extent of growth and expansion of this tumor. Natural androgenic, estrogenic, and cortical hormones all possess common steroid structural characteristics and have all been isolated from adrenal cortical tissue. This would not be unexpected, since embryologically the adrenal cortex and the gonads originate in the genital ridge. Grossly and histologically it has been shown that the androgenic and estrogenic types of hormones are produced in gonadectomized animals (12, 13, 14, 32, 34, 35). The biochemical processes that cause or the products that follow these changes in the mouse are unanalyzed at present with one exception (6). The evidence of hormone production from the study of the accessory reproductive organs indicates that the adrenal tumors of the hybrid animals produce less clear-cut variation between estrogenic and androgenic hormone production than was found in the parental strains.

The differential response to hybridization shown in the size of the adrenal tumors produced and the evidence of hormone production from these tumors in the various F_1 crosses might be an indication that the hormone potentials of strains of mice vary, and perhaps a series of alleles or multiple nonallelic factors affecting hormonal potentials are responsible for the production of the tumors and resulting stimulation of the accessory reproductive organs.

Speculation is not limited in application to the consideration of the problems in the adrenals of the gonadectomized animals. There are clear indications of differences in basic endocrine balance in at least some of the series of intact hybrids—namely, the DBA × CE and CE × DBA animals. One is then faced with the question of the importance of the adrenal cortex in determining and maintaining endocrine balance. Christy *et al.* suggested that the adrenal cortex might be responsible for excessive estrogenic production in these intact and gonadectomized animals (2). The adrenal is no doubt still further implicated in various internal imbalances.

From the experiment reported here and from the investigations by Smith, Kirschbaum *et al.* (12,

13, 19, 21–24), it has become evident that the response to gonadectomy as well as some of the aging changes in the adrenals of intact animals varies in different strains. This would indicate that the response itself, or the entire endocrine balance in inbred strains, is due to genetic factors. While the time of occurrence of the response may vary because of nonchromosomal parental influence, the actual response itself appears to be more of a genic nature. Whether the gene actions affect the gland itself or whether the environment of the gland is more important in producing the response has in part been investigated. It seems evident that the genic nature of the adrenal cortical tissue itself is of importance in determining the cortical response to gonadectomy (18).

More intensive study of several of these inbred strains employing genetic methods may clarify some of the results that have been observed in this pilot experiment. Such a study, involving reciprocal backcrosses of these F_1 hybrids with their respective parental strains, is in progress and should furnish more information about the variation and the reasons for the variation observed in the various parental strains and in the F_1 hybrids. Perhaps this genetic type of experiment on endocrine factors with the use of other inbred strains such as NH, BALB and other sublines of strain A would further increase our understanding of the genetic and nongenetic mechanisms that act on the endocrine system.

SUMMARY

Reciprocal crosses of strains A, C3H, DBA, C57BL, CE, and YEL were made, and approximately 1,700 offspring were produced. Half of these were gonadectomized at 1–3 days of age; the rest were maintained as intact control animals. Animals were autopsied at 15 days, 1 month, and at monthly intervals through 24 months of age or beyond. Endocrine and accessory endocrine organs were studied histologically. These studies are reported.

Since this was a pilot experiment and only one animal of each type was examined at each specific age, few general conclusions can be drawn from the results. Several interesting indications of parental influence and genic influence were suggested by the data.

The primary facts were: (*a*) Characteristic aging changes (x-zone degeneration, lipochrome cell occurrence, benign tumor formation and hyalinization) were observed in the adrenals of intact animals. (*b*) Adrenal cortical carcinomas were found in gonadectomized animals of all the crosses with evidence of hormone production beyond that found in the parental strains. (*c*) Structural characteristics in the ovaries (retention and hyalinization of corpora lutea, cyst formation, lipochrome cell deposition, and tumor occurrence) of females of the different crosses were observed and described. (*d*) Structural changes in the uteri (cystic glandular hyperplasia, adenomyosis, and adenomatous hyperplasia) of intact and gonadectomized females were observed, studied intensively, and reported separately. (*e*) Pituitary abnormalities (basophilism, and basophile adenomas) occurring in some gonadectomized animals were described in detail in another report.

The data indicate that reciprocal cross differences were concerned most usually with the time of occurrence of various changes, i.e., (*a*) time of appearance of lipochrome in the adrenals of some of the intact animals, (*b*) time of occurrence of mammary tumors, (*c*) time of occurrence of adrenal cortical carcinomas of gonadectomized animals, (*d*) time of stimulation of the vaginal epithelium of gonadectomized females, and (*e*) development of mammary gland rudiments in males.

Differences due to genes were more difficult to define, since they were all probably due to the combined action of several genes or alleles of genes. The indications are: (*a*) in CE hybrids the size of the adrenal, its structure, and the severity of the changes in it following gonadectomy were closely allied with the gene contribution from strain CE. (*b*) Occurrence of carcinoma in the adrenals of the gonadectomized hybrids was dominant or epistatic to the occurrence of nodular hyperplasia only and was also dominant to the absence of change. (*c*) Characteristic variations in the ovary, such as the lipochrome cell deposition, hyalinization, and the occurrence of cysts apparently also depend on gene action.

Some speculations on the aspects of hormonal balance and inheritance were made and the value of further investigation of the genetics of the endocrine relationships was discussed.

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The Interaction of Carcinogenic Hydrocarbons with Tissue Constituents

I. Methods*†

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INTRODUCTION

In spite of the enormous amount of experimentation that has been carried out with carcinogenic hydrocarbons, relatively little attention has been paid to the interactions between these substances and the susceptible tissues. By means of fluorescent microscopy, Graffi (4), Ahlström (1), and Simpson and Cramer (13) have observed a characteristic distribution of various hydrocarbons within the cells of epidermal tissue, with some concentration in the mitochondria of the perinuclear region. These observations, although of considerable cytological interest, provide little evidence as to the chemical identity of the fluorescent compound seen within the cell, the chemical makeup of the cellular material with which the hydrocarbon or its metabolites may be associated, the nature of the association, or the effect of this association on the biological and chemical function of the cellular constituent directly involved in the process.

Doniach *et al.* (3) and, more recently, E. C. Miller (10) have shown that benzpyrene derivatives are chemically bound to epidermal protein following the application of this carcinogen to mouse skin. These findings have a distinct parallel in those of Miller and Miller (11) on azo dye carcinogenesis, in which they demonstrated the occurrence of protein-bound dyes and their correlation with liver carcinogenesis.

The recent investigations on the metabolism of C^{14}-labeled carcinogenic hydrocarbons have provided new quantitative data on the distribution and excretion of these substances and have led to the identification of new metabolites (5, 7, 17). The phenomenon of binding of hydrocarbon to protein has been confirmed by these technics (6). This paper is the first in a series devoted to a comprehensive biochemical study of the interactions of radioactive hydrocarbons with cellular constituents of susceptible and resistant tissues. The investigation has so far included two tissues, mouse skin and mouse submaxillary gland, both of which have been shown to be susceptible to the carcinogenic action of the polycyclic aromatic hydrocarbons. The aim of these studies is to gain further information about the mechanism of carcinogenesis.

METHODS

Preparation of tissues for analysis.—Some difficulty was encountered in homogenizing mouse skin, but this could be decreased considerably by removing from the whole skin much of the fat and muscle of the dermis. This was accomplished as follows: The skin, shaved free of hair with surgical electric clippers, was stretched and pinned in place with the epidermal side against a plane surface (usually the flat side of a cork stopper). The cork and skin were then immersed in liquid air and frozen. The skin was next removed from the liquid air, and, after waiting about 30 seconds for the fatty layer to soften to the right degree, the muscle and fat of the dermis were flaked off with a scalpel.[1] The remaining skin (see Figs. 1 and 2) consisted of the epidermis and dermis containing the hair follicles and sebaceous glands. In spite of the heterogeneity of cell types present in the tissue, this selection was considered advantageous because of the probable involvement of the follicle cells and sebaceous glands in the carcinogenic process (12). After removal of the fat and muscle, the skin was finely minced with scissors and homogenized 5 minutes, with the media described be-

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[1] This method was originally developed by Dr. G. C. Mueller of the McArdle Memorial Laboratory.

low, in a Potter-Elvehjem homogenizer made of a thick-walled glass tube and a glass pestle.

Both lobes of the submaxillary gland were used. Prior to homogenization, the glands were stripped of fatty tissue and separated from the adjacent lymph nodes. They were then freed of connective tissue by passage through a small metal tissue mincer. The mince was caught in a tared receptacle, weighed, and transferred quantitatively to a Potter-Elvehjem homogenizer equipped with a plastic pestle. All analyses made on skin and submaxillary gland tissues were based on the wet weight of minced tissue.

Nucleic acid and protein nitrogen analyses.—Homogenates of skin and submaxillary gland or cell fractions of these two tissues were analyzed for ribonucleic (RNA) and desoxyribonucleic (DNA) acids by Schneider's hot trichloroacetic acid technic (15). Nitrogen determinations were made on the protein remaining after the extraction of the nucleic acids using the micro method described by LePage (9). It was found from the difference in tissue weight before and after treatment with hot trichloroacetic acid that the procedure, in addition to extracting the nucleic acids, also resulted in the solubilization of some protein. Therefore, the values obtained in this study do not give the total protein nitrogen of the tissues or tissue fractions.

Cell counts.—The number of cells contained in a given weight of minced mouse skin was determined by counting the nuclei present in an aliquot of the homogenized mince. To accomplish this, the minced tissue was allowed to stand overnight in enough ice-cold 3 per cent acetic acid to make a 2.0 per cent suspension, after which procedure it could be homogenized easily in a Potter-Elvehjem homogenizer made of a thick-walled glass tube and a plastic pestle. Homogenates prepared in acetic acid were superior to those made in sucrose or saline, because the acid fixed the nuclei and softened the keratin, thus permitting use of the plastic pestle. This treatment minimized the possibility of rupturing nuclei and obviated the presence of small particles of glass in the homogenate which usually occurred when a glass pestle was used. The nuclei, stained with gentian violet, were counted in a hemocytometer by the standard procedure for the enumeration of white blood cells (8). Cell counts were obtained on homogenates of mouse submaxillary gland in a similar manner. The results of the nucleic acid and protein analyses are given in Table 1.

Cell fractionation.—Several attempts were made to fractionate mouse skin into cellular components with differential centrifugation. Although the tissue could be homogenized in 0.154 M KCl, 0.25 M sucrose, or 0.88 M sucrose, centrifugation of these homogenates at even low speeds invariably gave only two fractions: the particulate matter consisting of whole cells, nuclei, mitochondria and other cytoplasmic granules; and a clear supernatant composed of soluble proteins. In the experiments involving the labeled hydrocarbon, the separation was carried out at 600×*g*.

The first attempt to separate mouse submaxillary gland into cellular components involved homogenization of the tissue in 0.25 M sucrose and fractionation by differential centrifugation into nuclear, large granule, small granule, and supernatant fractions. Nucleic acid analyses on the cell fractions obtained showed that 70 per cent of the DNA was present in the supernatant fluid, indicating that the nuclei were ruptured during either homogenization or centrifugation. Similar analyses on nuclear and cytoplasmic fractions obtained from homogenates prepared in media containing nuclear fixatives showed that essentially all the DNA was in the nuclear fraction. This demonstration of nuclear fragility made it important to select a medium which would "harden" the nuclei and at the same time permit the isolation of the cytoplasmic constituents. The medium described by Schneider and Petermann (14) involving the addition of calcium chloride was used successfully. Nuclei prepared in this medium contained virtually all the DNA originally present in the whole tissue. Microscopic examination of the nuclei showed them to be transparent and to have no precipitated chromatin. The nuclear fraction was somewhat contaminated with cytoplasmic granules, and there appeared to be a slight tendency for these granules to clump together. However, it was possible to remove all the cytoplasmic contamination from the nuclear fraction by washing several times with 0.5 M sucrose. This washing did not result in any loss of DNA from the fraction.

TABLE 1

CELL COUNTS, NUCLEIC ACID AND PROTEIN NITROGEN ANALYSES ON MOUSE SKIN AND SUBMAXILLARY GLAND

Tissue	DNA mg/gm wet tissue	RNA mg/gm wet tissue	Protein N mg/gm wet tissue	Nuclei/gm wet tissue ×10⁻³	DNA/cell μg.×10⁶	RNA/cell μg.×10⁶
Skin	3.93±0.45	1.19±0.25	15.7±1.9	5.74±0.40	7.1	2.2
Submaxillary gland	5.83±0.29	8.80±0.86	14.1±1.0	4.37±0.27	13.4	20.1

The method finally adopted for the cellular fractionation of mouse submaxillary gland is outlined in Charts 1 and 2. After mincing, the tissue was transferred to a Potter-Elvehjem homogenizer with 0.88 M sucrose-0.0018 M $CaCl_2$ to make a 10

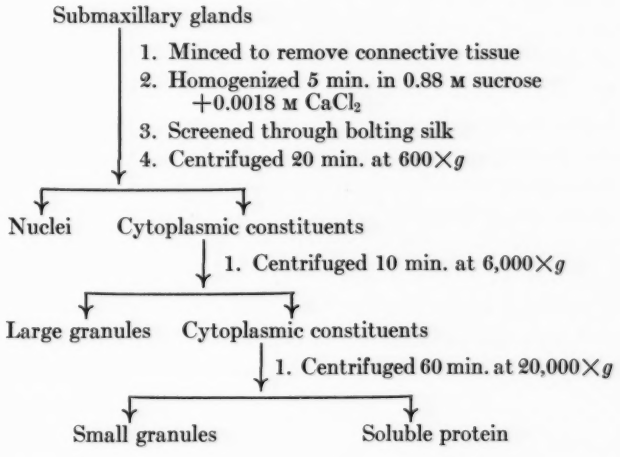

Submaxillary glands
1. Minced to remove connective tissue
2. Homogenized 5 min. in 0.88 M sucrose +0.0018 M CaCl2
3. Screened through bolting silk
4. Centrifuged 20 min. at 600×g
Nuclei
Cytoplasmic constituents
1. Centrifuged 10 min. at 6,000×g
Large granules
Cytoplasmic constituents
1. Centrifuged 60 min. at 20,000×g
Small granules
Soluble protein

CHART 1.—Cell fractionation scheme

per cent suspension, and it was homogenized for 5 minutes. To remove bits of whole tissue and unbroken cells, the homogenate was then forced through one thickness of Swiss Bolting silk fastened over the delivery end of a 1-ml. tuberculin syringe. In this way, up to 6 ml. of homogenate could be screened without any

appreciable loss, except for the bits of whole tissue held back by the silk. The supernatant fractions indicated in Chart 2 were combined prior to removal of the large granules. Cell fractionations have been carried out successfully on as little as 150 mg. of minced tissue, which is the amount obtained from two adult mice. The nucleic acid and protein analyses for the individual cell fractions are given in Table 2.

Application of labeled dibenzanthracene to skin.—Female Rockland mice were given an application

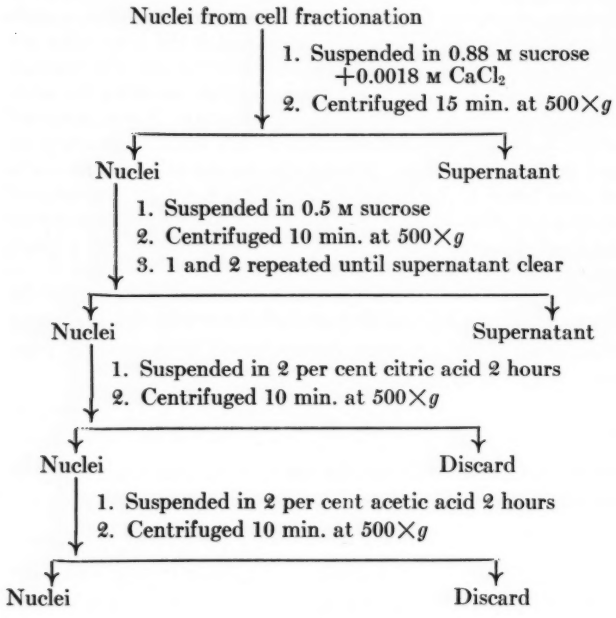

Nuclei from cell fractionation
1. Suspended in 0.88 M sucrose +0.0018 M $CaCl_2$
2. Centrifuged 15 min. at 500×g
Nuclei
Supernatant
1. Suspended in 0.5 M sucrose
2. Centrifuged 10 min. at 500×g
3. 1 and 2 repeated until supernatant clear
Nuclei
Supernatant
1. Suspended in 2 per cent citric acid 2 hours
2. Centrifuged 10 min. at 500×g
Nuclei
Discard
1. Suspended in 2 per cent acetic acid 2 hours
2. Centrifuged 10 min. at 500×g
Nuclei
Discard

CHART 2.—Purification of nuclei

of a solution of 1,2,5,6-dibenzanthracene-9,10-C^{14} in benzene on the back. The mice were killed, and the areas of skin to which the hydrocarbon had been applied were excised and rinsed with benzene to remove surface dibenzanthracene. The sections of skin were then freed of the subcutaneous tissue by the liquid air technic, weighed, minced, and homogenized in 0.154 M KCl, and an aliquot of the homogenates was assayed for C^{14} present. The homogenates were then transferred to 15 ml. graduated centrifuge tubes. The soluble proteins were separated from the particulate fraction by centrifugation at 600×*g* and were decanted carefully into clean centrifuge tubes. The particulate material was suspended in 5 per cent trichloroacetic acid, and the soluble proteins were precipitated by the addition of an equal volume of 10 per cent trichloroacetic acid. The proteins were allowed to remain in contact with the acid overnight in the refrigerator; then they were sedimented by centrifugation and washed by suspension, centrifugation, and decantation in the following solvents: 3 times in 95 per cent ethanol at room temperature, 3 times in 3:1 ethanol-ether solution at 60° for 5 minutes, and twice in ether at room temperature.

Counting technics employed.—The trichloroacetic acid solutions decanted from the precipitated proteins were discarded, as they were repeatedly shown to contain no radioactivity. The alcohol-ether washes from each protein sample were combined, and an aliquot was pipetted directly on aluminium discs and counted. The proteins were air-dried, weighed, and plated on aluminium discs which had a fine circular groove of known diameter punched into their surface. The powdered protein was suspended in 25 per cent ethanol on the plate and spread evenly to fill the area circumscribed by the groove and was dried in a vacuum desiccator. The protein adhered tightly to the plates. In determining the radioactivity of the protein samples, the necessary corrections were made for self absorption. Aliquots from various homogenates and cell fractions were assayed for radioactivity by wet combustion with the Van Slyke reagent (16), and the barium carbonate obtained from these combustions was plated on aluminum discs and counted (2). Corrections were made for self-absorption. All radioactive samples were counted either in internal-flow Geiger counters or internal-flow proportional counters which were used in conjunction with the Berkeley Decimal, G-M Scalers equipped with automatic timing devices. Counting of samples was continued long enough to give a statistical accuracy of at least 10 per cent.

TABLE 2

NUCLEIC ACID AND PROTEIN NITROGEN ANALYSES ON CELL FRACTIONS OBTAINED FROM SUBMAXILLARY GLAND HOMOGENATES PREPARED IN 0.88 M SUCROSE-0.0018 M $CaCl_2$

Cell fraction	DNA mg/gm wet tissue	RNA mg/gm wet tissue	Protein N mg/gm wet tissue
Whole homogenate	5.83	8.80	14.1
Nuclei	3.86	0.98	1.41
Acid wash of nuclei	0	0.25	0.96
Large granule	0.35	1.41	2.78
Small granule	0.11	4.14	4.46
Supernatant	0.05	0.67	1.83
Total*	4.37	7.45	11.44

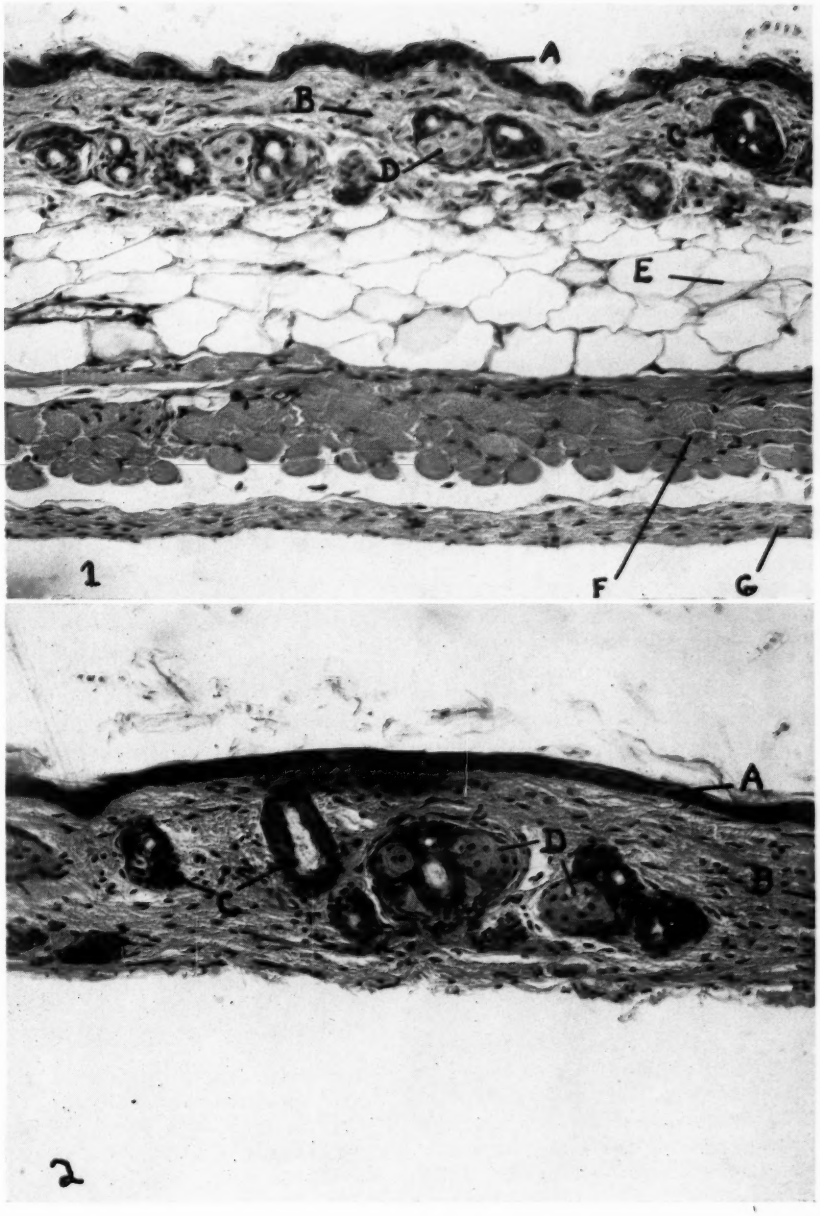
* Totals are somewhat lower than values for whole homogenate because of the loss of tissue particles held back by the bolting silk.

Injection of labeled dibenzanthracene into submaxillary glands.—The mice were anesthetized by

FIG. 1.—Intact skin from the back of a mouse.

FIG. 2.—Skin from the back of a mouse with the subcutaneous tissue removed following freezing in liquid air.

(*A*) epidermis, (*B*) connective tissue, (*C*) hair follicles, (*D*) sebaceous glands, (*E*) adipose tissue, (*F*) and (*G*) muscle.


A
B
C
D
E
F
G
1
A
D
B
C
2

the intraperitoneal injection of 0.15–0.20 ml. of a solution containing 10 mg. of Nembutal per ml. This dosage was equivalent to 9 mg/100 gm body weight. Because of the very narrow margin between the effective dose and the lethal dose, care was taken to use the smallest volume possible to effect anesthesia. The ventral side of the neck was then shaved with surgical clippers, and a vertical incision about 1.5 cm. long was made to expose the glands. The hydrocarbon was administered with the aid of a 0.25-ml. tuberculin syringe and a ½-inch No. 27 needle. One injection was made in the distal end of the gland, and the hydrocarbon was distributed throughout the gland as thoroughly as possible by partially withdrawing the needle and re-inserting it repeatedly in both lobes. The amount of liquid delivered depended on the vehicle used for the carcinogen. When a solution of the hydrocarbon in tricaprylin was used, 0.04 ml. could be injected; or, if a suspension of the hydrocarbon in 75 per cent sucrose was used, up to 0.12 ml. could be injected. The incision was closed with two metal wound clips and covered with a layer of collodion.

SUMMARY

Procedures have been developed for the homogenization of mouse skin and submaxillary gland. This has made possible the separation of submaxillary gland by differential centrifugation into its cellular components and also the determination of the number of cells, the nucleic acid content, and the quantity of protein nitrogen per unit of tissue. Methods have been described for the administration of labeled carcinogenic hydrocarbons to these tissues and for the determination of radioactivity in the isolated fractions and proteins.

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The Interaction of Carcinogenic Hydrocarbons with Tissue Constituents

II. 1,2,5,6-Dibenzanthracene-9,10-C^{14} in Skin*†

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Many investigators of the mode of action of the carcinogenic hydrocarbons have selected skin as the most suitable tissue for study. As a result, considerable information has been collected pertaining to epidermal carcinogenesis. Carruthers (6) has given the following reasons for regarding skin as the ideal tissue for the study of carcinogenesis: "(*a*) the carcinogen can be applied externally in a uniform manner with fairly uniform results; (*b*) the cells, which are of a single type arranged in layers of increasing differentiation, can be obtained practically free from contamination with cells other than those of epidermal origin; (*c*) the tissue lends itself readily to chemical analysis, for which it is suited because it is avascular, and therefore free from interference by blood; and (*d*) some features in the development of the cancer can be followed with the naked eye from the first application of the carcinogen through the hyperplastic or precancerous stage to the time of first appearance of cancer." In addition, the application of the hydrocarbon to the surface of the skin results in contact of the majority of the cell population within the treated area with the carcinogenic agent. The combination of these factors makes the use of skin especially desirable in a chemical investigation of tissue-carcinogen interaction.

The binding of derivatives of benzpyrene to epidermal protein has been demonstrated by Miller (11). It appeared desirable to extend this work to other carcinogens and with other methods. We have therefore undertaken this study by means of the tracer technic, using 1,2,5,6-dibenzanthracene-9,10-C^{14}, which has been successful in the determination of rates of excretion (8) and in the identification of new metabolites (7, 10, 16). It is unfortunate that none of the methods currently available for separating tissues into cell components could be applied successfully to mouse skin (15).

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METHODS

Ten female Rockland[1] mice were each given 0.1 ml. of a 0.5 per cent solution of dibenzanthracene-9,10-C^{14} in benzene, applied with a micro-pipette to a shaved area, 4–4.5 sq. cm., on the back. This dose is equal to 0.5 mg. of hydrocarbon, or 6.5×10^5 counts/minute/mouse. The mice were placed together in one cage and allowed to move freely. Two mice were killed with ether after 2 hours, 2, 9, 18, and 42 days, and were treated individually by the methods described in the preceding paper (15).

RESULTS

Time study.—The results of the time study of the interaction of dibenzanthracene-9,10-C^{14} with the particulate and soluble protein fractions of treated skin (the liquid-air preparation [15]) are tabulated in Table 1. Two hours after the application of the carcinogen, considerable radioactivity was present within the skin, and 5–6 per cent of this was chemically bound to protein. The radioactivity within the skin reached a maximum on the second day, and the amount of radioactivity bound to protein was greatest at this time. It is evident that from the second to the eighteenth day approximately 50 per cent of the radioactivity in the homogenate was bound. Some C^{14} could still be detected, both free and bound, on the 42d day.

It was also shown that the washing procedure was sufficient to remove all the adsorbed radioactive compounds. The residual C^{14} remained tightly bound to the protein and could not be re-

[1] Obtained from Arthur Sutter Co.

moved by boiling with benzene for 10 minutes or by continuous extraction of the proteins with boiling dioxan for as long as 20 hours, as shown in Table 2.

Mouse No. 11 in Table 1 served as a control to establish the extent of *in vitro* binding occurring during homogenization. The skin was removed from this animal before the administration of any hydrocarbon, and the tissue was prepared as in other cases, except that 0.15 mg. of dibenzanthracene-9,10-C^{14} (200,000 c.p.m.) was added as an aqueous colloid during the homogenization. The tissue was then fractionated and washed as usual. Under these conditions no *in vitro* binding occurred with the particulate protein, and only a relatively small amount of the total C^{14} present in the soluble protein could not be removed by washing.

Chart 1 was obtained by plotting the specific activities of the two tissue components against time. This curve is similar to one published by E. C. Miller, using fluorometric analysis of hydrolysates, for the binding of benzpyrene derivatives to mouse epidermis (11).

Reprecipitation of proteins.—In order to demonstrate more conclusively that the association between the radioactive compound and the tissue protein is indeed an irreversible chemical binding, the proteins were dissolved in suitable solvents, washed with organic solvents, reprecipitated, and their radioactivity again measured. A decrease in specific activity following this treatment would indicate dissociation of the C^{14} from the protein.

The soluble protein fraction was readily dissolved at room temperature in 98 per cent formic acid and reprecipitated by the addition of 5 volumes of 10 per cent trichloroacetic acid (TCA). The proteins of the particulate fraction were insoluble in formic acid but slowly dissolved in 5 N alcoholic potassium hydroxide. In this experiment 20 mg. of protein was suspended in 6 ml. of alcoholic potassium hydroxide and allowed to stand overnight at room temperature. At the end of this time, the undissolved protein was sedimented by centrifugation. The clear supernatant solution was decanted, the soluble protein precipitated by the addition of concentrated trichloroacetic acid, and

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN TREATED SKIN FOLLOWING ADMINISTRATION OF DIBENZANTHRACENE-9,10-C^{14}

Animal	Time after administration	Whole homogenate c.p.m.	Particulate fraction EtOH-Et_2O wash solution c.p.m.	Particulate fraction Protein c.p.m.	Soluble protein fraction EtOH-Et_2O Wash solution c.p.m.	Soluble protein fraction Protein c.p.m.
1	2 hrs.	4,600	3,400	160(5)*	1,800	90(13)*
2		6,500	5,600	240(7)	2,400	180(10)
3	2 days	7,900	3,000	3,140(55)	1,600	400(47)
4		11,000	8,800	990(47)	800	190(49)
5	9 days	2,400	350	880(21)	300	100(13)
6		1,200	130	580(15)	150	90(21)
7	18 days	1,600	500	340(10)	50	30(6)
8		1,000	200	500(12)	50	70(8)
9	42 days	800	200	80(2)	250	10(1)
10		600	200	80(2)	50	20(3)
11	0	200,000	55,500	40(1)	140,000	90(22)

* Numbers in parentheses are specific activities, c.p.m/mg.

TABLE 2

RADIOACTIVITY OF PARTICULATE PROTEIN FRACTION FOLLOWING EXTRACTION WITH BOILING SOLVENTS

Solvent used	Before extraction (c.p.m.)	Before extraction (c.p.m/mg)	After extraction (c.p.m.)	After extraction (c.p.m/mg)
Benzene*	1,046	10	1,040	10
Benzene*	1,083	11	1,122	12
Benzene*	1,740	26	1,673	25
Dioxan†		79		79

* Boiled 10 minutes.
† Continuous reflux for 20 hours.

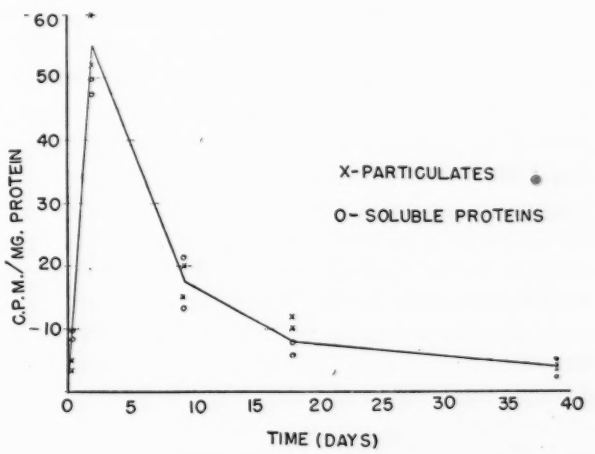

CHART 1.—Protein-bound radioactivity in skin fractions

about 3 mg. of precipitated protein was obtained by centrifugation. After reprecipitation, both protein fractions were washed with alcohol and ether, plated as previously described, and counted. The results are given in Table 3. The constancy of specific activity clearly shows that the radioactivity is truly bound to protein and rules out the possibility of surface absorption.

Nucleic acid extraction.—To determine whether the C^{14} was combined with nucleic acids, the washed proteins were extracted with 5 per cent trichloroacetic acid at 90° C. for 15 minutes. This procedure extracts and hydrolyzes nucleic acids

TABLE 3

REPRECIPITATION OF SKIN PROTEINS

Tissue fraction	Washed protein (c.p.m/mg)	Protein precipitated from HCOOH (c.p.m/mg)	Protein precipitated from KOH solution (c.p.m/mg)
Particulate	12		13
Soluble protein	20	22	
" "	20	19	

oughly with benzene to remove any hydrocarbon adhering to the surface of the skin. Fat and muscle were removed from the skins by the liquid air technic. The skins were pooled, minced finely with scissors, and homogenized in 5 volumes of 0.14 M NaCl-0.01 M phosphate buffer, pH 6.8. The nucleoproteins were extracted and purified by a modification of the method of Mirsky and Pollister (13). Two aliquots were removed for C^{14} determination. The homogenates were then stirred for 2 hours, centrifuged, and the supernatant fluid decanted. Washing of the tissue with buffered saline was continued until no more TCA precipitable protein was extracted. Ribose nucleoprotein was removed with this washing. Protein soluble in this buffered saline but not precipitable by acidification to pH 4 with acetic acid (which precipitates the RNA-protein) was precipitated by the addition of TCA, and is referred to as "soluble protein."

The solid residue remaining after the extraction of the ribose nucleoprotein was suspended in 1 M NaCl and stirred continuously overnight. The

TABLE 4

EFFECT OF HOT TRICHLOROACETIC ACID EXTRACTION ON RADIOACTIVE PROTEIN

Mouse no. and fraction	Protein before treatment			Protein after treatment		
	Weight	c.p.m.	c.p.m/mg	Weight	c.p.m.	c.p.m/mg
1-Part.*	31.6	157	5	14.4	59	4
2-Part.	35.2	244	7	13.6	81	6
3-Part.	57.5	3,140	55	30.9	1,610	52
4-Part.	20.4	985	47	9.8	585	60
5-Part.	42.0	875	21	13.7	274	20
6-Part.	39.6	578	15	21.6	324	15
1-Sol. prot.†	6.6	86	13	5.0	43	9
2-Sol. prot.	7.1	179	25	5.1	51	10

* Particulate fraction.
† Soluble protein fraction.

(14). The proteins were then dried, plated, and counted. Table 4 shows the results of these determinations. In most cases the specific activities of the proteins remained constant through this treatment, proving that the radioactivity is not firmly bound to nucleic acids. The total radioactivity in each sample was reduced, but this was due to the loss of protein solubilized by the extraction procedure. Accurate determination of the radioactivity in these solutions was not possible because of the high dilution of radioactivity caused by the presence of large quantities of trichloroacetic acid.

Extraction of nucleoprotein from mouse skin.—Four female mice were each painted with dibenzanthracene-9,10-C^{14} over the major portion of the back. Each mouse was given 0.2 ml. of a solution of the hydrocarbon in benzene equivalent to 0.5 mg. (600,000 c.p.m.). The mice were allowed to run freely in the same cage. At the end of a week, the mice were killed with ether. The skins were removed from the backs and were washed thor-

desoxyribose nucleoprotein present in the supernatant following centrifugation at 2,000 × *g* for 60 minutes was precipitated by dialysis against 0.14 M NaCl for 12 hours. Purification of the nucleoprotein was accomplished by redissolving it in 1 M NaCl and reprecipitating it by dialysis as before. The insoluble protein residue from the 1 M NaCl extraction was termed "insoluble protein."

The four protein and nucleoprotein samples were each washed 3 times with ethanol at 25°, 3 times with 3:1 ethanol-ether at 60° for 5 minutes, and twice with ether at 25°. The proteins were then plated and counted, and the results are shown in Table 5. Thus, although no C^{14} is bound to nucleic acid, there is an appreciable binding to nucleoprotein.

DISCUSSION

The experiments reported here confirm and extend the work of Miller (11), who proved for the first time that derivatives of 3,4-benzpyrene are

bound to epidermal protein of mice. The analytical procedure developed by Miller involved the measurement of fluorescence following alkaline hydrolysis of the thoroughly extracted protein preparation. The application of the tracer technic to this problem has provided an accurate and direct measurement of the quantity of dibenzanthracene and its metabolites in skin and the fraction bound to protein. This determination (although the fluorescence method gives considerable information about the structure of the compound) does not depend on hydrolytic conditions and complete extraction of the liberated substances, and, hence, may be considered to give more rigorously quantitative data. Experiments with the labeled dibenzanthracene have further demonstrated, in agreement with Miller's (11) findings with benzpyrene, that the bound material is not produced as an artifact *in vitro* during homogenization of the skin fraction in the presence of the carcinogen, but results from some vital process occurring within the cells.

An appreciable quantity of radioactivity bound to skin protein was found as early as 2 hours after application of the carcinogen. The highest concentration was observed at 2 days, as compared to 1 day (11) in the case of benzpyrene. The subsequent decrease results from two factors. First, because the mice lick the hydrocarbons off their backs (5), the quantity is rapidly decreased. Second, the amount of hydrocarbon present within the cells decreases as the compound is metabolized and excreted in the feces (7–9). It is possible that, if the mice were prevented from removing the dibenzanthracene from the site of application, the peak of bound material might occur at a later time. It would be of considerable interest to determine what effect preventing the mice from licking the hydrocarbon would have on the incidence and induction time of tumors. Nevertheless, since it has been shown (1) that tumors can be induced by a single application of a carcinogenic hydrocarbon followed by promotion with croton oil, it would appear that studies at an early time after administration would provide the most cogent information about the mechanism of hydrocarbon carcinogenesis.

The fact that the maximum protein-bound material is observed within 2 days, in agreement with the observations of Miller (11), lends credence, according to this type of reasoning, to the belief that the protein binding may play a causal role in carcinogenesis. This has been effectively demonstrated by the Millers (12) in the case of the azo dyes. Indeed, it is almost axiomatic that a chemical carcinogen should initiate by a chemical mechanism the complex and, as yet, obscure sequence of events leading to malignancy. An interaction, such as that observed here, with a tissue constituent essential somehow to the control of growth would appear to be a necessary prerequisite for such a process of carcinogenesis.

Boyland (3) has postulated that binding would occur between carcinogenic hydrocarbons and tissue groups exposed to them, and our observations provide experimental support for this idea. More recently, however, he has expressed a belief (4), based on the *in vitro* enhancement of the solubility of carcinogenic (and other) hydrocarbons by aqueous purine solutions, that there is an association between the carcinogen and the purine moieties of the desoxyribonucleic acid within the cell nucleus.

TABLE 5

SPECIFIC RADIOACTIVITY OF NUCLEOPROTEIN FRACTION

Total activity in homogenate = 39,000 c.p.m.

Sample	Sp. Act. c.p.m/mg
Soluble protein	24
RNA protein	31
DNA protein	23
Insoluble protein	22

He is of the opinion that this binding is relatively weak, being somewhat "stronger than the linkage of a substrate to an enzyme" (2). The prolonged presence of the carcinogenic complex within the chromosomes, he feels, would eventually lead to mutations that would give rise to tumors.

Our work with labeled dibenzanthracene and that of Miller (11) were undertaken to investigate, by means as direct as possible, the interaction of a carcinogenic hydrocarbon and a susceptible tissue. It is our belief that this direct, empirical method offers a sounder approach to the mechanism of chemical carcinogenesis than do physical-chemical investigations of model systems, whose analogies to the situation obtaining *in vivo* are often tenuous at best.

The experiments here reported do not disprove the postulates of Boyland, but they do show that up to 50 per cent of the total hydrocarbon and metabolites present within the cells of the skin is bound not as a weak complex but instead by a strong chemical bond. The irreversible nature of this binding is indicated by the fact that the radioactivity associated with the tissue cannot be removed by prolonged washing with organic solvents, and, more important, by the fact that these proteins can be dissolved under appropriate conditions, washed with organic solvents, and reprecipitated without any decrease in specific activity. These experiments further show that the binding

we observe does not occur with nucleic acids, since these compounds can be removed without decreasing the specific activity of the tissue. These findings are very similar to those made earlier (12) for the azo dyes.

It is of interest that the protein binding was found in nucleoprotein, particulate, and soluble proteins. At present it is not possible to decide which, if any, of these proteins are important in carcinogenesis, and we cannot elucidate the question of whether the initiation of cancer involves primarily a nuclear or cytoplasmic mechanism at this time. A more detailed visualization of the means by which the protein-bound derivative of the hydrocarbon might act has been presented by Miller (11).

It will remain for future work to determine the identity of the bound compound (which we provisionally believe to be the "true" carcinogen). Miller (11) has shown that neutral and acidic fluorescent substances are liberated by hydrolysis from epidermal protein following administration of benzpyrene. Other important considerations are the type of linkage through which the hydrocarbon or its metabolite is held to the protein, the nature and function within the cell of the proteins involved, and the attempt to correlate more securely this phenomenon with carcinogenesis. Such a correlation has been achieved with signal success with the azo dyes (12). The beginning of a similar correlation has been made by Miller (11) between the binding of benzpyrene to protein and the eventual appearance of tumors. She found the binding primarily in the epidermal fraction, the site at which tumors eventually arise, and lower levels of bound hydrocarbon were found in the epidermis of mice exposed to light, which parallels the lower incidence of tumors obtained under similar conditions. Investigations along these lines are continuing in this laboratory.

SUMMARY

The irreversible chemical binding of 1,2,5,6-dibenzanthracene-9,10-C^{14} to the proteins of a skin fraction containing epidermis and part of the dermis has been demonstrated. The variation of specific activity of the protein with time after application has been determined. The binding occurs only *in vivo* and is not due to surface adsorption. The binding occurs between the carcinogen or its metabolite and the nucleoproteins, particulate and soluble proteins, but not with nucleic acids. The possible significance of this phenomenon in the carcinogenic process is discussed.

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The Interaction of Carcinogenic Hydrocarbons with Tissue Constituents

III. 1,2,5,6-Dibenzanthracene-9,10-C^{14} in the Submaxillary Gland*†

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INTRODUCTION

The desirability of extending the study of the interaction of 1,2,5,6-dibenzanthracene-9,10-C^{14} with cellular components of susceptible tissues made necessary the selection of a more suitable tissue than mouse skin. Mouse submaxillary gland was chosen, as it is susceptible to the carcinogenic action of hydrocarbons and can easily be separated into cell components (11).

The first demonstration of the carcinogenic susceptibility of this tissue was made by Rusch, Baumann, and Maison (7), who produced squamous-cell carcinomas by the injection of benzpyrene dissolved in corn oil into the surgically exposed glands of mice. Tumors developed more rapidly in this gland than in any other tissue that had been tested. Ten tumors were obtained in fifteen strain A mice with an induction time of from 2 to 3.5 months. Steiner (10) obtained a tumor yield of 67 per cent by implantation of a pellet composed of 1 mg. of dibenzanthracene and 1 mg. of cholesterol after surgical exposure of the gland. The first tumor was produced in 4 months, and the histological classification of most of these tumors was the same as that found by Rusch *et al.* (7). When methylcholanthrene (10) was used in the pellet, a wider variety of tumors was produced, but the tumor incidence and the induction time were about the same as with the dibenzanthracene. Similar experiments have been carried out by implanting crystals of methylcholanthrene (3) and pieces of dibenzanthracene dispersed in beeswax (2). In our hands the results of tumor induction experiments were very similar to those obtained by the workers already mentioned.

METHODS

The technic of injection of the hydrocarbon solutions and of fractionation of the gland into cellular components is described in paper I (11). In the experiment with tricaprylin, each of fifteen female Rockland mice was injected with 0.04 ml. of a solution which contained 0.3 mg. of dibenzanthracene (400,000 c.p.m.). In the experiments with sucrose, the hydrocarbon was finely powdered and suspended in 75 per cent sucrose; 0.10 ml. was injected into each of 56 mice. The dose of hydrocarbon was the same as in the tricaprylin experiments. It was not possible to obtain an even distribution throughout the gland.

RESULTS

Experiment with tricaprylin.—Three mice were killed by ether at 2 hours, 2, 7, 27, and 35 days after injection, and the glands were removed and pooled. Homogenization of the tissue and its separation into cellular components were carried out according to methods previously described (11), and the proteins in each fraction were precipitated and washed with organic solvents. Radioactivity was determined in the whole homogenate, in each of the cell fractions, in the organic washes, and in the washed protein. Table 1 gives the results obtained 1 week after the injection of the hydrocarbon. The large granules at this time contained about 40 per cent of the total radioactivity present in the homogenate. In each of the fractions 0.2–0.3 per cent of the radioactivity present remained bound to the proteins after washing with organic solvents. This bound radioactivity could not be removed by extraction with 5 per cent trichloro-

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acetic acid (TCA) at 90° for 15 minutes or by reprecipitation from 98 per cent formic acid or performic acid (following extraction with organic solvents) as shown in Table 2. In most of the cases presented and in many others studied, the specific radioactivity of the proteins remained constant.

Similar data to those recorded in Table 1 were obtained for each of the five time intervals. Chart 1 consists of the plots of the total radioactivity contained in the whole homogenate and the total radioactivity bound to protein in each of the cell fractions versus time.

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN SUBMAXILLARY GLAND

(One week after injecting DBA-C^{14}-1.2 × 10^6 c.p.m. in tricaprylin)

Cell fraction	Total C^{14} (c.p.m.)	Protein-bound C^{14} Total activity (c.p.m.)	Protein-bound C^{14} Specific activity (c.p.m/mg)
Homogenate	530,000		
Nuclei	73,000	110	19
Large granules	205,000	675	58
Small granules	23,000	35	9
Soluble protein	104,000	290	27

TABLE 2

STABILITY OF PROTEIN-BOUND RADIOACTIVITY

Cell fraction	Washed protein (c.p.m/mg)	Protein extracted with hot TCA (c.p.m/mg)	Protein precipitated from HCOOH (c.p.m/mg)	Protein precipitated from HCOOOH (c.p.m/mg)
Nuclei	16	17	15	15
Large granules	44	42	42	29
Small granules	25	24	20	23
Soluble protein	22	22	23	17

The true representation of the change in radioactivity present in the whole homogenate with time would be a curve with its highest point at zero time and having a negative slope reflecting the metabolism of the hydrocarbon and its excretion from the gland. The increase in radioactivity contained in the whole homogenate, shown in Chart 1, illustrates the difficulty of removing the glands from the animals at early time intervals without loss of oil. This difficulty is especially marked 2 hours after injection. After about 1 week, the oil solution is encapsulated within the gland, allowing removal of the gland without loss of oil, so that after 7 days the curve for the whole homogenate shows the proper negative slope.

While the initial phases of the curves representing the bound radioactivity in the cell fractions may be influenced by this leakage, certain trends are evident over the remainder. The radioactivity present in the large granule and nuclear fractions decreases with time, while that in the small granules and soluble protein fractions is increasing. It should be pointed out that the relative quantities of tissue distributed between the two granule fractions is not the same in the presence of the tricaprylin as is obtained when oil is not present. When oil is present in the homogenate, there is an increase in the amount of tissue sedimented in the large granule fraction and a concomitant decrease in the quantity of small granule tissue as compared to the values obtained previously (11). As the amount of oil in the tissue diminished during the experiment, the relative quantities of tissue in the two fractions returned to the normal values. Since the changes in the bound radioactivity contained in the two granule fractions may be due to variations in the amount of tissues contained in each, interpretation of the curves is complicated.

Experiments with sucrose suspensions.—Fifty-six Rockland female mice were injected. Eight mice

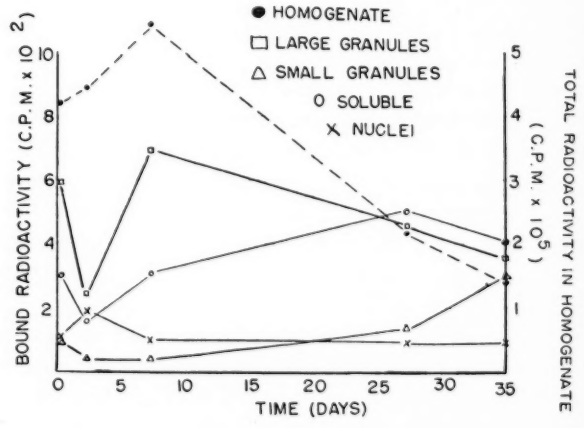

CHART 1.—Radioactivity in cell fractions of submaxillary glands injected with tricaprylin solutions of dibenzanthracene-9,10-C^{14}.

were killed with ether at each time interval after the injection; the glands were removed and pooled into two groups of four each. Because of the presence in the glands of some of the sucrose suspension, there was some loss of hydrocarbon during their removal. The two groups of tissues were homogenized and fractionated, and the proteins in each cell fraction were precipitated with TCA and washed with organic solvents. The proteins were dried in a vacuum desiccator and weighed. They were then extracted with 5 per cent TCA at 90° for 15 minutes and were washed once each with 5 per cent TCA, 95 per cent ethanol, absolute ethanol, and ether. They were dried the second time in a vacuum desiccator and dissolved in 98 per cent formic acid, extracted with benzene and precipi-

tated with TCA. Finally, the proteins were dissolved in performic acid (6), made from 1 ml. of 98 per cent formic acid to which was added 0.2 ml. of 30 per cent hydrogen peroxide, were extracted with benzene, and were incubated at 30° for 2 hours. At the end of this time, the protein was precipitated with TCA, washed with alcohol and ether, plated, and counted. Although the data given in Table 2 indicate a possible decrease by performic acid in the amount of C^{14} in two of the four cell fractions, it was felt that reprecipitation from this solvent represented the most rigorous demonstration of chemical binding; therefore, it was used routinely in this experiment.

TABLE 3

DISTRIBUTION OF RADIOACTIVITY IN SUBMAXILLARY GLAND

(One week after injecting DBA-C^{14} in sucrose)

Cell fraction	Total C^{14} (c.p.m.)	Protein-bound C^{14} Total activity (c.p.m.)	Protein-bound C^{14} Specific activity (c.p.m/mg)
Whole homogenate	870,000		
Nuclei	309,000	49	11
Large granules	74,000	94	21
Small granules	15,000	126	20
Soluble protein	215,000	337	19

The animals were killed eight at a time after 2 hours and on the 2d, 7th, 14th, 21st, 28th, and 35th days; the tissue was divided into two groups. By the second day, the sucrose solution was completely absorbed, leaving crystals of hydrocarbon localized at three or four points within the gland.

In Table 3 is recorded the total radioactivity present in the whole homogenate and the distribution of radioactivity among the cell fractions 1 week after the injection of the carcinogen. In contrast to the results obtained from the tricaprylin experiments, the greatest concentration of radioactivity appeared in the nuclear fraction instead of the large granule fraction. This undoubtedly resulted from the fact that the crystals of hydrocarbon still present in the gland were sedimented with this fraction; therefore, the large percentage of radioactivity in the fraction is probably due to contamination.

The distribution of C^{14} in each of the cell fractions and the total radioactivity present in the homogenate obtained *in vivo* are plotted against time in Chart 2. Once again, some loss of hydrocarbon was observed during removal of the glands at 2 hours, but a rapid increase in C^{14} present in the soluble protein fraction was observed at 7 days. This change in the relative amount of C^{14} present in the soluble protein fraction is also evident in the plot of total bound radioactivity against time, seen in Chart 3. These variations in total and bound radioactivity present in the soluble proteins indicate that events are occurring *in vivo* which represent a response of the hydrocarbon to the tissue, or vice versa.

Attention should also be given to the fact that, although the highest concentration of total radio-

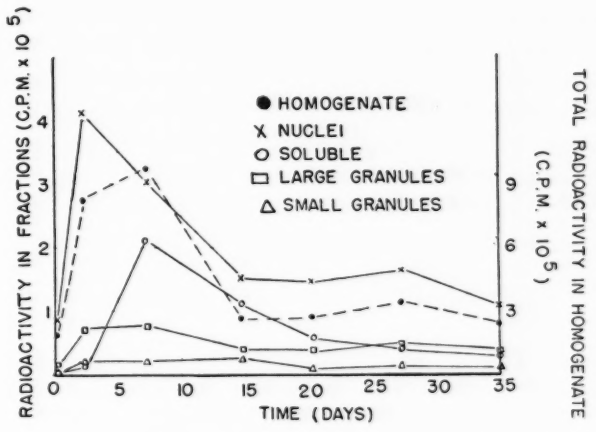

CHART 2.—Total radioactivity in cell fractions of submaxillary glands injected with a sucrose suspension of dibenzanthracene-9,10-C^{14}.

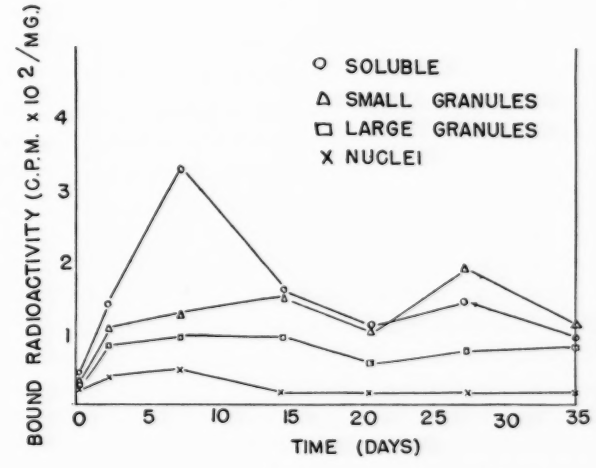

CHART 3.—Specific activity of proteins in cell fractions of submaxillary glands injected with sucrose suspensions of dibenzanthracene-9,10-C^{14}.

activity was present in the nuclear fraction throughout the experiment (Chart 2), the amount of C^{14} bound in this fraction is the lowest of the four (Chart 3). It is felt, therefore, that this latter chart represents the relative capacity of each of the fractions to bind the hydrocarbon, which might represent a measure of the response of the cellular component to the hydrocarbon. The presence of considerably more bound radioactivity in

the cytoplasmic granules and soluble proteins is in agreement with the results of the fluoromicroscopic studies mentioned in the introduction to paper I (11).

In vitro *experiments.*—The fact that crystals of the hydrocarbon were localized at only a few places within the gland (despite efforts to obtain a more thorough dispersion) makes it questionable whether sufficient cells came in contact with the carcinogen *in vivo* for its effects to be detectable by the chemical methods employed. Furthermore, it appeared possible that the distribution of radioactivity observed in these experiments resulted from contact of the cell components with the carcinogen during homogenization and not from contact *in vivo.* Thus, it became necessary to compare the distribution of radioactivity *in vitro* with that found at the different time intervals throughout the experiment. To accomplish this, an experiment was carried out in which the suspension of labeled hydrocarbon in sucrose was added to fresh minced glandular tissue and homogenized immediately. The tissue was then separated into cell fractions, and the protein in each fraction was precipitated, washed with organic solvents, extracted with hot TCA, and reprecipitated from performic acid. The distribution of radioactivity among the fractions and the amount of binding in each fraction are recorded in Table 4. These results parallel closely the distribution obtained *in vivo* at 2 hours and 2 days after the administration of the carcinogen, but not at longer times.

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN SUBMAXILLARY GLAND FOLLOWING THE *in Vitro* APPLICATION OF DBA-C^{14} IN SUCROSE

Cell fraction	Total C^{14} (c.p.m.)	Protein-bound C^{14} Total activity (c.p.m.)	Protein-bound C^{14} Specific activity (c.p.m/mg)
Whole homogenate	400,000		
Nuclei	312,000	77	32
Large granules	66,000	179	18
Small granules	8,000	25	10
Soluble protein	3,000	24	3

Several experiments have been carried out to investigate further the character and the extent of *in vitro* binding occurring under conditions comparable to those present during homogenization and fractionation of glands exposed to the hydrocarbon *in vivo.* In one of these experiments, 700 mg. of minced submaxillary glands were separated into cellular components, and the tissue in each fraction, suspended in the sucrose-$CaCl_2$ medium, was divided into two equal parts. One group of four fractions, consisting of nuclei, large and small granules, and the soluble protein, was heated on the steam bath for 10 minutes. These four fractions were then cooled to 3° C. A solution of chromatographically pure dibenzanthracene-9,10-C^{14} in tricaprylin was added to each fraction in the group, and the same quantity of the oil solution was added to each cell fraction of the tissue which was not heated. The oil was then thoroughly dispersed throughout the tissue in each of the eight fractions by working the pestle of a Potter-Elvehjem homogenizer up and down several times through the suspensions. The proteins in each of the fractions were then precipitated with TCA, washed with organic solvents, extracted with hot TCA, and plated in the way described for *in vivo* experiments. The results of this study are given in Table 5. Binding of radioactivity to protein occurred in all the fractions, and similar results were obtained whether the tissues were fresh or heated. However, it is significant that the specific activities of the proteins from the *in vitro* experiments were lower in all cases than those obtained from a comparable *in vivo* experiment, as shown in Tables 1 and 5.

TABLE 5

BINDING OF RADIOACTIVITY TO CELL FRACTION PROTEINS OF MOUSE SUBMAXILLARY GLAND FOLLOWING THE *in Vitro* ADDITION OF DBA-C^{14} TO EACH FRACTION IN TRICAPRYLIN

Cell fraction	DBA added (c.p.m.)	Specific activity of fresh tissue (c.p.m/mg)	Specific activity of boiled tissue (c.p.m/mg)
Nuclei	67,000	7	7
Large granules	167,000	11	10
Small granules	67,000	7	2
Soluble protein	167,000	6	7
Whole liver	334,000	0	

Hydrolytic experiments.—The first of these experiments demonstrated the effect of dilute alcoholic potassium hydroxide on proteins obtained from large granule and soluble protein fractions. The proteins were dissolved in 1:2.5 ethanol:0.1 N KOH solution (1.4 ml/10 mg of tissue). One sample was heated at 45° C. for 30 minutes, and the other was heated at 80° C. for 30 minutes. Following this treatment, the proteins were cooled to room temperature and precipitated with TCA. They were then washed with ethanol, absolute ethanol, and ether, and finally were plated and counted. The results, given in Table 6, show that treatment of the large granule protein at the lower temperature had no effect on the specific activity of the protein; however, heating the soluble protein at the higher temperature caused a 42 per cent decrease in specific activity.

Another experiment was carried out to determine the effect of drastic alkaline and acid hydrolysis on radioactive proteins. One sample of protein weighing 40 mg. was dissolved in 2.5 ml. of 4 N KOH. To the solution was added 1.5 ml. of ethanol and 1 ml. of toluene, and the mixture was refluxed for 2 hours. At the end of this time no protein was precipitable with TCA, indicating that the hydrolysis had reduced the protein molecules to short chain peptides and free amino acids. The solution was cooled and extracted 5 times with 2–3 ml. of benzene. The aqueous solution was then acidified with concentrated HCl and again extracted with the same quantity of benzene. These benzene solutions were dried with sodium sulfate and their volumes reduced to 0.4 ml. Aliquots of each (0.2 ml.)

TABLE 6

EFFECT OF DILUTE ALCOHOLIC KOH ON RADIOACTIVE PROTEINS

Cell fraction	Temperature of heating	Specific activity: Before treatment (c.p.m/mg)	Specific activity: After treatment (c.p.m/mg)
Large granule	45° C.	6	8
Soluble protein	80° C.	17	10

were plated directly on aluminum plates and counted. Finally, the acidified aqueous solution was extracted thoroughly with butyl acetate, and this extract was plated and counted. The results of the experiment are given in Table 7. Acidic and neutral compounds were extracted from the reaction mixture, but the sum of the radioactive compounds present in the benzene solutions and in the butyl acetate accounted for only 39 per cent of the radioactivity originally present in the protein.

A second sample of protein weighing 24 mg. was dissolved in 4 ml. of 48 per cent hydrobromic acid and was refluxed on a sand bath for 2 hours. No protein could be precipitated from the solution at this time. The solution was cooled and extracted 5 times with 2–3 ml. of benzene. The benzene solution was then extracted with 1 N NaOH. After acidification of the alkali with concentrated HCl, the acidic compounds present were re-extracted into benzene. The hydrobromic acid solution was further extracted with butyl acetate. The organic solutions were dried with sodium sulfate and reduced in volume. Aliquots of the solutions were then plated, and their radioactivity was determined. The results of this experiment are also tabulated in Table 7.

Extraction of nucleoprotein from mouse submaxillary gland (in vivo).—Thirteen female Rockland mice were each injected in the submaxillary gland with 0.04 ml. of a solution of 1,2,5,6-dibenzanthracene-9,10-C^{14} in tricaprylin. Each mouse was given 0.03 mg. (40,000 c.p.m.) of the hydrocarbon. The mice were killed 1 week later, and the glands were removed. The glands were cleaned of fat and lymphatic tissue and homogenized in 7 ml. of 0.14 M NaCl-0.01 phosphate buffer, pH 6.8 for 5 minutes. The methods of extracting the ribose and desoxyribonucleoproteins were the same as those described for the extraction from skin (12), and the terms "soluble" and "insoluble" proteins have the same meaning as given them in that paper.

The four protein and nucleoprotein samples were washed with organic solvents as described for skin and were then dissolved in performic acid and incubated with this reagent at 37° C. for 2 hours. The proteins were precipitated with TCA and washed with 95 per cent ethanol, absolute ethanol, and ether. Then they were plated and counted, and the results are shown in Table 8.

TABLE 7

EFFECT OF DRASTIC HYDROLYSIS ON RADIOACTIVE PROTEINS

Hydrolytic medium	Initial activity of protein (c.p.m.)	Acidic derivative extracted by benzene (c.p.m.)	Neutral derivative extracted by benzene (c.p.m.)	Derivatives extracted by butyl acetate (c.p.m.)
Alcoholic KOH	460	47	44	87
48 per cent HBr	190	8	41	16

TABLE 8

RADIOACTIVITY OF NUCLEOPROTEINS

Sample	Bound C^{14} (c.p.m/mg): *In vivo*	Bound C^{14} (c.p.m/mg): *In vitro*
Soluble protein	3	2
DNA protein	11	14
RNA protein	9	15
Insoluble protein	11	10
Total radioactivity present	370,000 c.p.m.	400,000 c.p.m.

Extraction of nucleoprotein from mouse submaxillary gland (in vitro).—This experiment was a duplication of the *in vivo* experiment as regards the extraction technics employed. Glands from six female Rockland mice were removed and minced. This tissue was homogenized in the presence of 0.04 ml. of 1,2,5,6-dibenzanthracene-9,10-C^{14} dissolved in tricaprylin (400,000 c.p.m.) in 0.88 M sucrose-0.0018 M $CaCl_2$ for 5 minutes and worked up as described above. The results are shown in Table 8.

DISCUSSION

The demonstration that dibenzanthracene or its metabolites is chemically bound to the proteins of mouse submaxillary gland, a tissue particularly susceptible to carcinogenesis, is another step in the

correlation of this phenomenon with the mechanism of tumor induction. The demonstration by the Millers (5) that azo dyes are bound to rat liver proteins has been extended by the observation that acetylaminofluorene is also bound to liver proteins (5). In the carcinogenic hydrocarbon series, benzpyrene (5) and dibenzanthracene (12) are known to bind to skin protein. It seems possible that, since this reaction has been observed with several classes of chemical carcinogens, a common mechanism of tumor genesis may obtain. Such a mechanism, based on sound experimental evidence, might well apply to "endogenous" human cancer. Once sufficient information is available about the details of the process of carcinogenesis, it might be possible to devise a new mode of tumor therapy or prophylaxis.

It was hoped at the outset that submaxillary gland might be an ideal tissue for studies on the mechanism of hydrocarbon carcinogenesis. The fact that it could readily be fractionated into its cellular components and that changes of radioactivity with time could be ascertained lent promise towards a fuller interpretation of the biochemical processes involved in the malignant change. It was found that protein binding did occur, and bound radioactivity was found in all the cell fractions in the gland. However, there were no systematic changes with time, which might lead to a definite interpretation of the importance of a given cell fraction, and the experimental difficulties in obtaining a uniform distribution of the carcinogen throughout the gland militated against effective interpretation.

It has already been stated that the tricaprylin, used as the solvent in one series of experiments, influenced by flotation the quantity of proteins in the individual fractions. However, it is definite that at early times about 40 per cent of the radioactivity in the gland accumulated in the large granules. This result is similar to those obtained for skin by Ahlström (1) and Graffi (4). They found that the greatest concentration of fluorescence was associated with the mitochondria and that the remainder was present in other cytoplasmic granules or diffused throughout the cytoplasm. They observed no fluorescence within the nuclei. Their observations and our total radioactivity distribution very probably result from the dissolution of the hydrocarbon in intracellular lipids, known to be present primarily in the mitochondria. The gross distribution of the hydrocarbon injected in sucrose was primarily in the nuclei and was undoubtedly due to the occlusion of crystals on particles which were sedimented according to their mass. The fact that by both modes of administration most of the hydrocarbon was localized in a few places, and did not contact a majority of the cells, makes interpretation of total radioactivity of little use.

A much better correlation of results obtained with both solvents can be made by consideration of the bound radioactivity. A review of Tables 1 and 3 and Charts 1 and 3 will show some similarities in the amount of hydrocarbon present in like fractions from the two experiments. This distribution indicates a characteristic affinity of each cell component for the hydrocarbon. Binding of the radioactivity does not result from the contamination of the protein by a residue of the total C^{14} originally present in the fraction. That this is true is particularly well illustrated by the fact that the nuclear fraction in the sucrose experiment contained the highest concentration of total radioactivity, due to the sedimentation of hydrocarbon crystals, but contained the lowest amount of bound isotope. This was true at every time interval studied. Thus, the binding in amounts characteristic of each cell fraction represents a measurable response of the tissue to the carcinogen.

Examination of Charts 1 and 3 shows no systematic changes of the radioactivity in the large and small granules with time. (The apparent changes in Chart 1 are due to the alterations in proportion of the two types of granules due to flotation with tricaprylin.) However, in both experiments, a significant change with time was observed in the protein-bound radioactivity in the soluble protein fraction. This observation is of particular significance in the sucrose experiment, where unchanged hydrocarbon was present in crystalline form. Considering the insolubility of the hydrocarbon in aqueous media and the distribution data obtained from the 2-hour, 2-day, and *in vitro* experiments (Chart 3 and Table 4), it seems necessary that this increase in bound and total radioactivity present in the soluble proteins should come from some water-soluble substance, a metabolite of dibenzanthracene. The subsequent decrease in radioactivity probably results from the elimination of the metabolite from the tissue. This finding is interesting in connection with the work of the Millers (5), who found that most of the azo dye bound to the soluble proteins of rat liver occurred in a single electrophoretic fraction (9), which fraction was much reduced in quantity in the tumors induced by the dye.

As in the case of skin (12) we have found that the hydrocarbon or its metabolite is not bound to nucleic acid, but is bound to nucleoprotein. The

decision as to whether carcinogenesis involves a nuclear or cytoplasmic mechanism (8) will have to be held in abeyance until more definitive experiments can be devised and executed. It is of interest that the amount bound to the nuclear fraction is small compared to the individual cytoplasmic fractions. Unfortunately, no conclusions as to a nuclear or cytoplasmic mechanism can be drawn from such quantitative comparisons.

It is also difficult at the present time to assess the significance of the *in vitro* binding of the carcinogen or to differentiate it completely from the binding *in vivo*. In most experiments the *in vitro* binding occurred to a smaller extent than that observed in a comparable *in vivo* experiment. Occasionally it did not occur at all, and on other occasions the amount of binding was comparable to that produced *in vivo*. This is in contrast to the consistent and reproducible results found in the living animals. Radioactive proteins obtained *in vitro* maintained constant specific activity through extractions with hot TCA and reprecipitation from hot formic and performic acids following benzene extraction of the protein solution. In this respect they were the same as the proteins obtained from the *in vivo* studies. The problem of differentiating the two must be given further consideration. However, if it can be shown conclusively that the property of a tissue to bind carcinogenic hydrocarbons is directly correlated to carcinogenesis, then the binding that occurs *in vitro* as well as *in vivo* must be a manifestation of the same phenomenon. Until further experimental data are available the temptation to speculate on the chemistry of the *in vitro* bound hydrocarbon will be resisted.

It would appear, although valuable information has been gained from these experiments with submaxillary gland, that until improved methods become available for the administration of the hydrocarbon so as to contact the majority of cells in the gland, further experimentation with this tissue would be of little use. Therefore, the main effort along these lines will be redirected to skin. Work is now in progress on the identification of the bound material and on developing correlations between tissue susceptibility to carcinogenesis and protein binding.

SUMMARY

The distribution of radioactivity following the direct administration of 1,2,5,6-dibenzanthracene-9,10-C^{14} into the submaxillary glands of mice has been studied. Tricaprylin and sucrose were used as the vehicles of injection. The distribution of total radioactivity depended on the vehicle. The presence of protein-bound radioactivity in all cell fractions of the gland was established. Only the C^{14} in the soluble protein fraction showed significant changes with time. The binding occurred with nucleoprotein, but not with nucleic acids. This binding could be obtained *in vitro*. The possible significance of these findings in relationship to the process of carcinogenesis has been discussed.

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Synergistic Action of Leukemogenic Agents*

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Ionizing radiations (4), carcinogenic hydrocarbons (12), and estrogenic hormones (3) are leukemogenic for mice of specific genetic constitutions (8). Synergistic and additive effects of x-rays and methylcholanthrene have been demonstrated (1, 13). A preliminary paper has been published on the synergistic action of x-rays and an estrogenic hormone (9).

In the present report genetic requirements for synergism are considered, and the synergistic action of estrogenic hormone and a carcinogenic hydrocarbon is demonstrated.

MATERIALS AND METHODS

BALB/c (Bagg albino), CBA, and DBA/2 mice, equally divided between the sexes (unless otherwise indicated), were used in these experiments. Test animals were progeny of untreated controls maintained as breeding stock by brother-sister matings.

Animals were 42 days of age, unless otherwise indicated, when the experimental procedure was instituted. General body irradiation was accomplished by exposing the mice in cardboard boxes at 30 cm. t.d., 140 kv., and 2 mm. aluminum filter, HVL=4.2 mm. Al. Methylcholanthrene was painted on the skin as a 0.25 per cent solution in benzene, a different site being utilized for each successive (3 times weekly) skin painting. Estrogenic hormone (estradiol dipropionate) was administered subcutaneously, either dissolved in peanut oil, or as a cholesterol pellet.[1]

Mice were observed until they developed lymphoid neoplastic disease, i.e., either mediastinal (thymic) lymphosarcoma or leukemia, at which time they were killed and autopsied. Animals dying from other causes were autopsied within 24 hours after death or when death seemed imminent.

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[1] Estrogenic hormone supplied by Ciba Pharmaceutical Products through the courtesy of F. W. Houghton.

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Specific experimental procedures will be described in connection with the report on results of each experiment.

OBSERVATIONS

BALB/c.—Mediastinal (thymic) lymphosarcoma did not appear spontaneously in 240 BALB mice. Lymphatic leukemia appeared infrequently before mice were 400 days of age. Leukemia, which was usually characterized by a very large spleen with macroscopic areas of lymphoid infiltration, occurred after 400 days more often in females (22 per cent) than males (4 per cent), although in either sex the incidence was no more than 1 per cent prior to the age of 400 days.

Induction of thymic lymphosarcoma and leukemia by estrogenic hormone in BALB mice.—Estradiol dipropionate, in the form of a 1–2-mg. pellet, was administered by trocar at 18, 36, or 72 days of age. Twenty-seven of 84 mice developed lymphomas, almost all thymic lymphosarcomas, before 400 days of age. The ultimate incidence of lymphoid neoplastic disease was at least 56 per cent (47 of 84 mice). Only males received pellets; females develop pyometra and do not survive long enough to develop leukemia.

Induction of thymic lymphosarcoma and leukemia by x-rays in BALB mice.—Three whole-body doses of 200 r given at weekly intervals caused seven cases of thymic lymphosarcoma to appear before 400 days of age in 32 irradiated mice.

Relative ineffectiveness of methylcholanthrene as a leukemogen for BALB mice.—Of 108 mice receiving 18 skin paintings (Chart 2, *b*), no cases of leukemia or lymphosarcoma appeared before 400 days of age. Of 35 animals receiving 36 skin paintings, three cases of lymphomatous disease (one thymic lymphosarcoma) developed prior to 400 days of age. This strain of mice, as contrasted with DBA/2 (Chart 4, *h*) is relatively, but not absolutely, resistant to the leukemia-inciting action of this carcinogenic hydrocarbon. The over-all incidence of leukemia was actually reduced because of the development of skin cancers.

Synergistic effects in BALB mice. X-rays and estrogenic hormone.—With a single dose of 200 r

whole-body, only two cases of leukemia appeared before 400 days of age in 97 mice (Chart 1, *a*), the ultimate incidence being 29 per cent. Only one case showed heavy thymic infiltration. This dose of x-rays is considered (at least before 400 days of age) only mildly leukemogenic.

When 5 μg. of estradiol dipropionate were given weekly in one subcutaneous injection for 14 successive weeks, beginning at 42 days of age, with no further treatment, three of 47 (Chart 1, *b*) developed lymphomas before 400 days of age. Although the ultimate incidence in this group was increased to 30 per cent (fourteen of 47), only three of the cases were thymic lymphosarcomas. Using the first 400 days of life as the period for calculating incidence, estradiol dipropionate was, in this dose, only very moderately leukemogenic.

When whole-body radiation with 200 r was followed immediately by the hormonal treatment, *sixteen cases appeared in 71 mice before they were 400 days of age* (Chart 1, *c*), and in fourteen the thymus was involved either exclusively or was very heavily infiltrated. When 400 r of x-rays was combined with the hormonal treatment (Chart 2, *e*), the synergistic effect was revealed more conclusively. By 300 days of age only one of 34 mice receiving radiation alone (Chart 2, *d*), and only one of the 47 receiving hormonal treatment (Chart 1, *b*) had developed leukemia, whereas in the group in which radiation was supplemented by hormone, twelve of 30 had developed lymphomas by 300 days of age (Chart 2, *e*), ten of these being thymic. The over-all incidence was 65 per cent, higher than that achieved by the administration of large doses of any single leukemogen.

If the 200 r of x-rays and the estrogen given in 5-μg. doses weekly had operated in only additive fashion, then in 71 animals no more than seven lymphomas should have appeared before 400 days of age. The actual number was sixteen.

Thymic radiation followed by estrogenic hormone (Chart 1, *f*).—When the entire body except the thymus was shielded during exposure to 200 r,[2] followed by 14 weeks of treatment with estrogen, six of 34 mice developed thymic lympho-

[2] Shielding effected by lead 2 mm. thick.

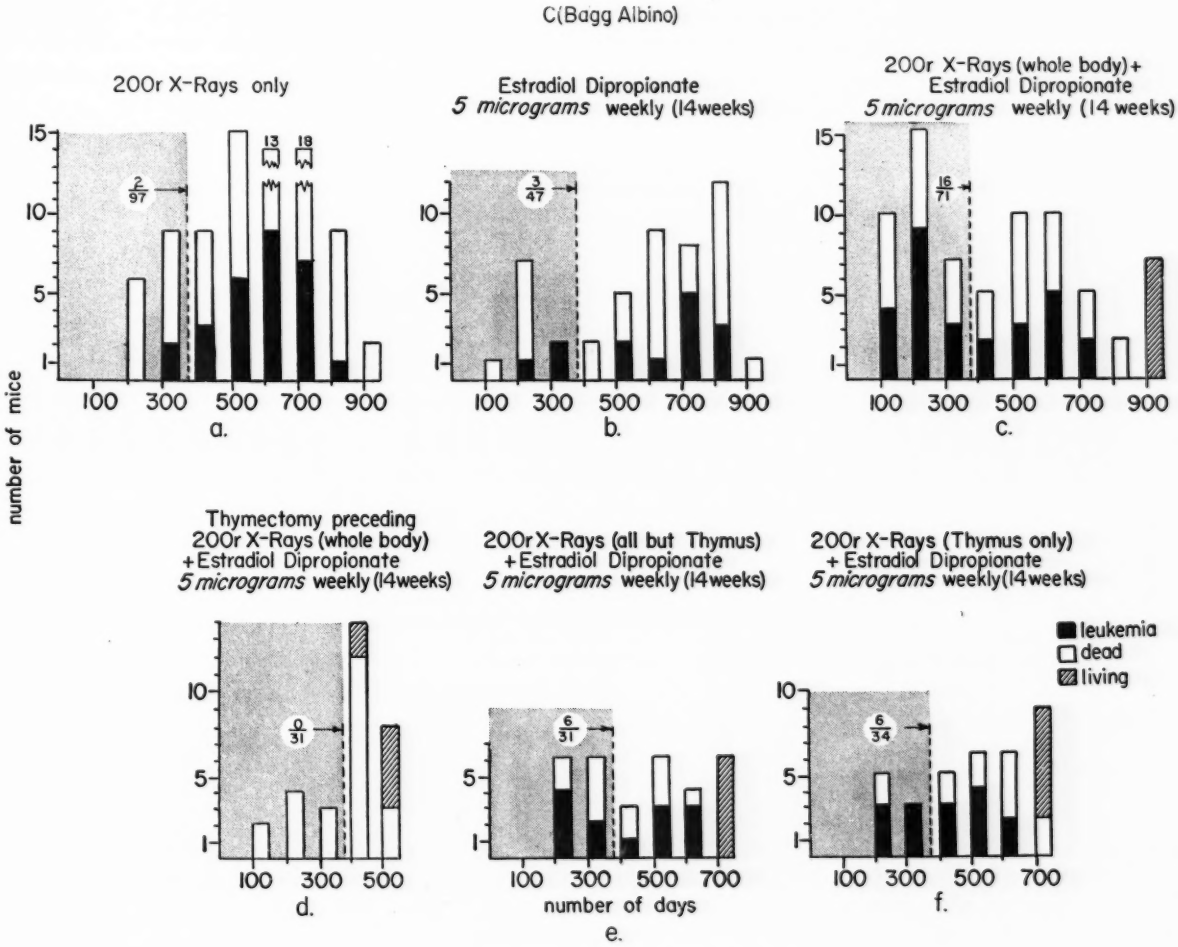

CHART 1

sarcomas by 400 days of age. Ultimately, thymic tumors appeared in eleven, whereas in 47 receiving the hormone alone only three thymic tumors were found (Chart 1, *b*). One thymic tumor was seen in 97 mice which received whole-body exposure to 200 r.

Radiation of the whole body except the thymus, followed by estrogenic hormone (Chart 1, *e*).—When the thymus was shielded during radiation with 200 r, and the 14-week treatment with estrogen was given, thymic tumors appeared in six of 31 mice by 400 days, although the thymus itself was not radiated. Although the total incidence of lymphomatous disease was only 39 per cent, as compared to 30 per cent in the group receiving hormone only, the onset of the disease was accelerated, and more cases (nine of 31 compared with three of 47) showed very large thymus glands.

Effect of thymectomy on leukemogenesis by synergistic action of x-rays and estrogenic hormone (Chart 1, *d*).—In thymectomized mice (urethan-anesthetized for thymectomy), the simultaneous leukemogenic action of x-rays and estrogen was abolished (none out of 31). Intact urethan-anesthetized controls developed thymic lymphosarcomas and leukemia to the same degree (seven out of 29 before 400 days), as did unanesthetized animals similarly treated (sixteen out of 71).

Lack of synergistic effect of x-rays and methylcholanthrene in BALB mice.—Eighteen skin paintings of 0.25 per cent methylcholanthrene caused no leukemias or lymphosarcomas to appear before 400 days in 102 mice so treated (Chart 2, *b*). In contrast, six of 131 DBA mice which received eighteen skin paintings developed lymphomas by 300 days of age (Chart 4, *c*), while only three cases appeared in 269 controls of similar age (Chart 4, *a*) As indicated above, 200 r of x-rays is mildly leukemogenic in BALB mice (Chart 2, *a*). When radiation with 200 r was followed immediately by the eighteen skin paintings (Chart 2, *c*), the incidence of lymphomas by 400 days was no greater (three of 98) than in mice receiving 200 r only (two of 97), nor was the total incidence of lymphomas increased. In DBA mice, however, x-rays and methylcholanthrene operated synergistically (Chart 4, *b*, *c*, *d*).

Combined action of methylcholanthrene and estradiol dipropionate in BALB mice.—Eighteen skin paintings of methylcholanthrene induced no

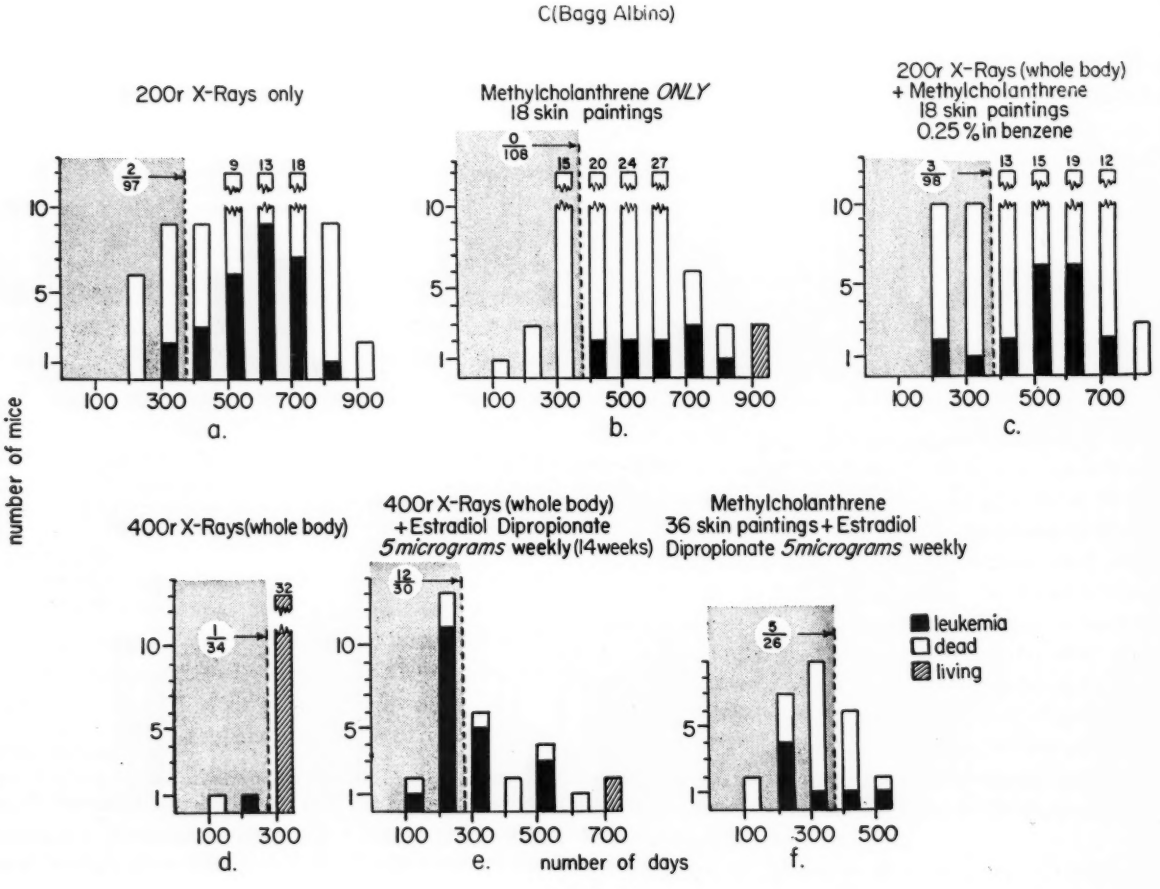

CHART 2

lymphomas in 102 BALB mice, but with 36 skin paintings, by 400 days of age, three of 35 animals developed lymphomas, one of which was a thymic lymphosarcoma. When combined with estradiol dipropionate, 36 skin paintings induced five thymic lymphosarcomas in 26 mice by 400 days (Chart 2, *f*). This is about twice the expected incidence if the effect were only additive, but it is questionable whether true synergism resulted. By contrast, in the DBA strain when eighteen skin paintings (Chart 4, *c*, six of 131 by 300 days) were combined with 5 μg. weekly of EDP (Chart 4, *e*, one of 47), seventeen of 55 had developed lymphomas (Chart 4, *f*). In this case, if the effect had been merely additive the expected incidence would have been 3.5, whereas the actual incidence was seventeen of 55.

CBA mice.—As in the BALB strain, spontaneous leukemia appears more frequently in females (fifteen of 105) than males (six of 112). Only one thymic lymphosarcoma appeared spontaneously in the 217 controls. Only three of the lymphomas (leukemia or lymphosarcoma) appeared before 400 days of age.

Induction of leukemia with large doses of leukemogenic agents in CBA mice.—In general, the response of CBA mice to the leukemogenic agents paralleled that observed in BALB mice. When pellets of estrogenic hormone were implanted at 70 days, five thymic lymphosarcomas had appeared by 400 days of age in fifteen mice, whereas in only three of 217 controls lymphomas had developed by this age. X-rays in heavy doses were likewise leukemogenic (13). Methylcholanthrene failed as a leukemogen. None of 38 with 36 skin paintings, and only one of 113 receiving seventeen skin paintings developed leukemia in the first 400 days.

Synergistic effects in CBA mice.—X-rays and estradiol dipropionate acted synergistically in increasing the incidence of lymphomatous disease in the first 400 days of life (Chart 3, *a*, *b*, *c*). If the 200 r of x-rays and the 5-μg. weekly dose of estrogen had acted in additive fashion, the expected incidence would have been about six in 49 mice, whereas actually it was seventeen (Chart 3, *c*). From the standpoint of total incidence, the effect was not so striking, since the total incidences of lymphomas were 23 per cent for 200 r, 45 per cent for estradiol dipropionate, and 55 per cent for the combination. In the last group the majority of the cases were thymic lymphosarcoma, in contrast to the first two. When the disease appears early in life, the thymus is heavily infiltrated. When 400 r

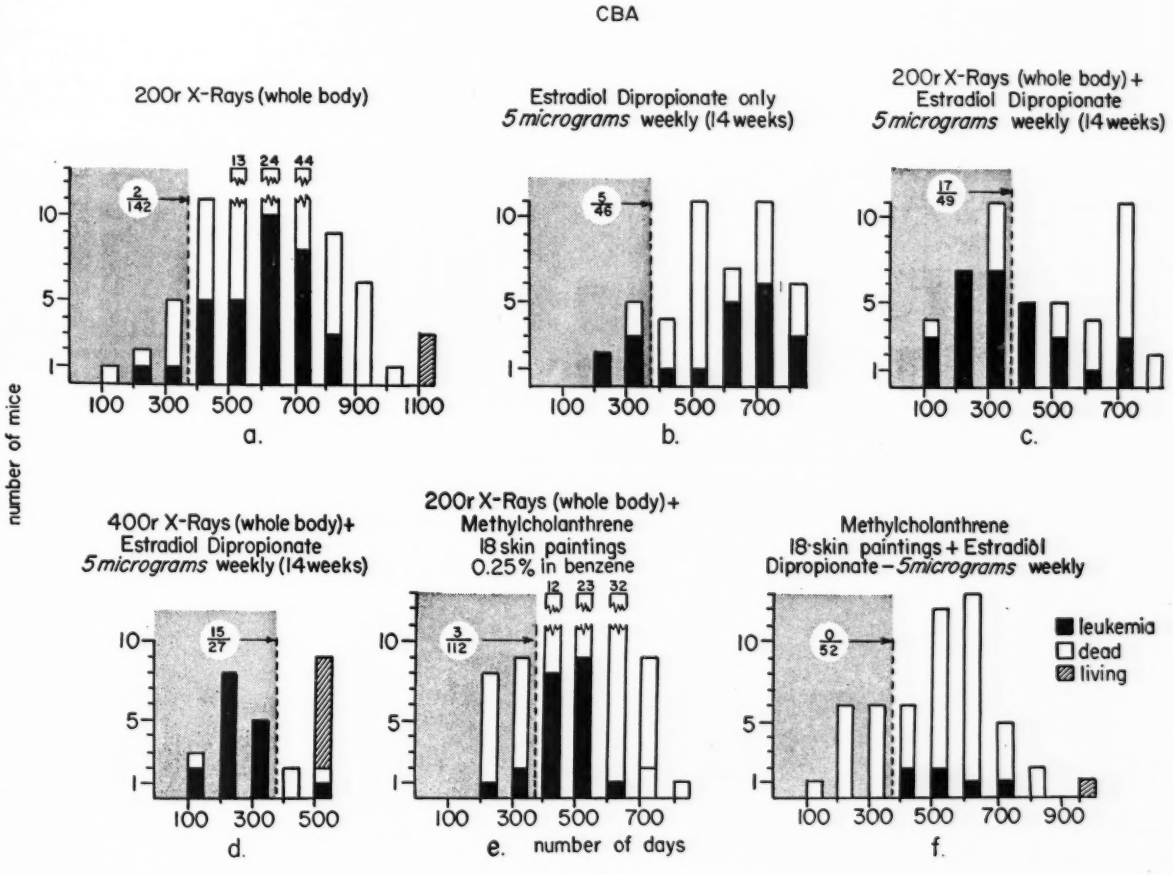

CHART 3

was combined with the estrogen, fifteen of 27 mice had developed lymphomas by 400 days (Chart 3, *d*). Combining x-rays and methylcholanthrene (Chart 3, *e*) or methylcholanthrene and estrogen (Chart 3, *f*) did not accelerate the onset of lymphomas.

The thymus and partial-body radiation with reference to induction of lymphomas in CBA mice.—Of 28 CBA mice which were thymectomized prior to receiving 200 r plus estrogen, no cases of lymphocytic neoplastic disease appeared. Two cases of chlorotic granulocytic leukemia appeared between 400 and 600 days of age. If only the thymus was radiated with 200 r, followed by 5 μg. of EDP for 14 weeks, lymphomas were not induced in high frequency (one in 32 before 400 days). If the entire body except the thymus was radiated and similar estrogenic treatment given, two of 32 developed thymic lymphosarcomas by 400 days of age, which is approximately the same incidence as that obtained with the estrogen alone (Chart 3, *b*). *Thus, it appears that in CBA mice partial-body radiation was not effective even when used together with estrogen in inducing lymphocytic neoplasia.*

DBA mice.—In this strain the spontaneous disease occurs more frequently in females (31 out of 146) than males (seventeen out of 123), although the age distribution is similar for the two sexes. Whether lymphomas occur spontaneously or are induced, the age of appearance is earlier in DBA's than in the other two strains. Consequently, 300 days of age was used as the base-line age for determining synergistic effects in accelerating the onset of lymphomas. Other respects in which the DBA's differ from the other two strains are the following:

1. In DBA mice, methylcholanthrene is leukemogenic (Chart 4, *a*, *c*, *h*).

2. Estradiol dipropionate decidedly potentiated the leukemogenic action of methylcholanthrene (Chart 4, *c*, *e*, *f*). With the dose of estradiol dipropionate used, no independent leukemogenic effect was obtained (Chart 4, *e*), whereas the addition of this dose to eighteen skin paintings of methylcholanthrene increased the incidence of lymphomas in the first 300 days from 4½ per cent (six out of 131) to 31 per cent (seventeen out of 55). The over-all incidence was increased from 20 to 46 per cent.

3. The accelerating effect of x-rays and methylcholanthrene was more than additive (Chart 4,

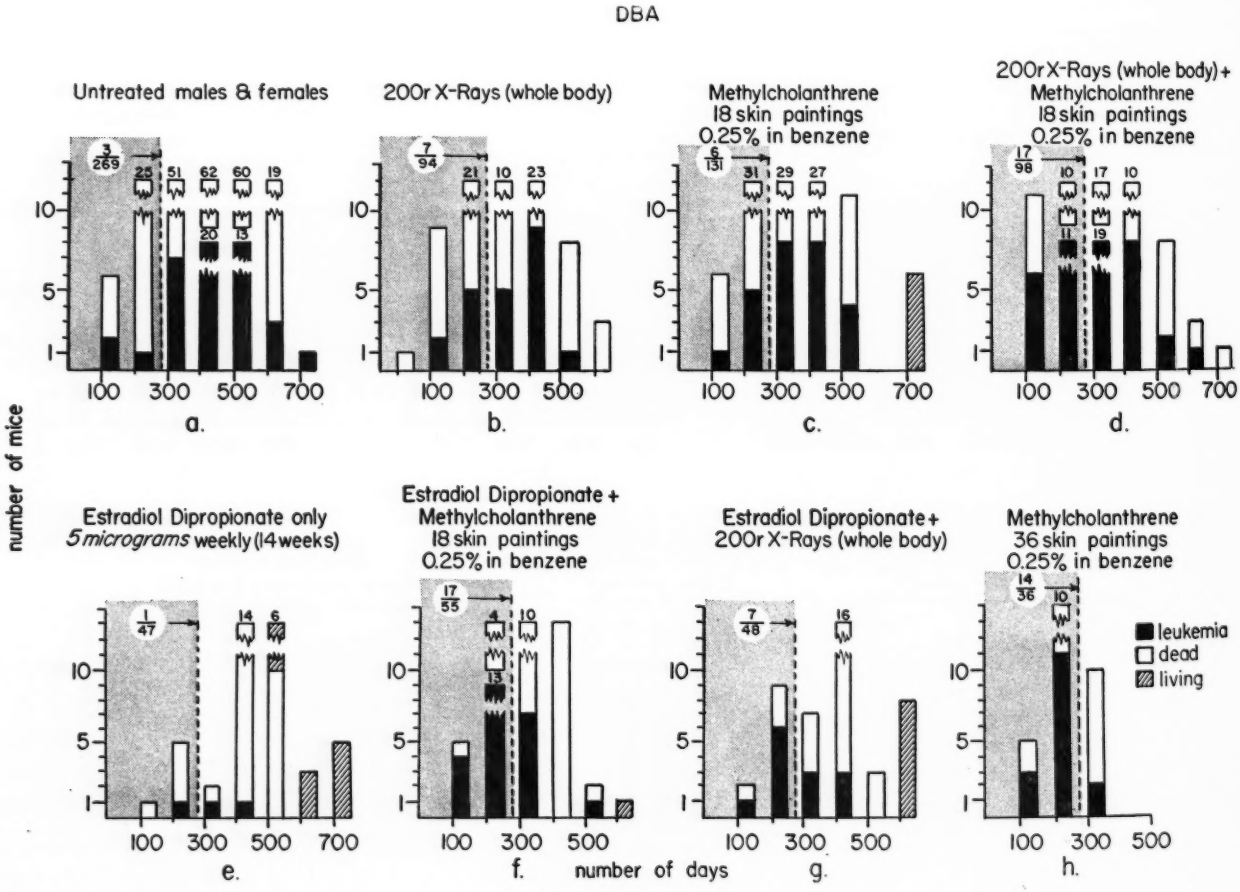

CHART 4

b, *c*, *d*), with seventeen cases appearing in 98 mice by 300 days, whereas the calculated incidence by addition of independent accelerating action is twelve. The over-all incidence exceeded that calculated by adding the incidences expected in 98 mice by the independent action of methylcholanthrene and x-rays (47–41).

As in the CBA's and BALB mice, estrogenic hormone potentiated the leukemogenic action of x-rays (Chart 4, *b*, *e*, *g*).

DISCUSSION

By their combined action agents may be more strongly leukemogenic than when used independently. This is especially true for the early induction of lymphomatous disease. If the incidence for the first 300–400 days of life is considered, then the combined action in accelerating onset is in many cases synergistic, i.e., the incidence exceeds the calculated additive expectancy.

The primary site of action for this accelerated development of lymphomas in young mice is the thymus. When this organ was removed prior to leukemogenic treatment, leukemia was not induced. In young mice only the thymus may be involved in the lymphomatous process, or the disease may be systemic with the thymus heavily infiltrated, suggesting that the initial involvement was in this organ. When leukemia appears in CBA or BALB mice beyond 500 days of age, even when the incidence is increased due to the leukemogenic activity of administered agents, the thymus is only very infrequently involved. Whether the thymus has served as the primary source of leukemic cells even in these older mice, without its being appreciably enlarged, is not known. If BALB or CBA mice receive leukemogenic treatment after thymic involution has taken place, then susceptibility is negligible.[3] The effect upon susceptibility in older mice of delaying thymic involution by gonadectomy is now being tested.

The importance of the thymus in mouse leukemogenesis (10, 11) is reaffirmed by these studies. It has been demonstrated that susceptibility to spontaneous (10) and carcinogen- (11) and x-ray-induced (5) leukemia is diminished by thymectomy. Estrogen-induced leukemia is characterized by a very large thymus (3), and thymectomy reduces the incidence of leukemia in mice subjected to the combined action of x-rays and estrogenic hormone, suggesting that the susceptible locus for the estrogenic-induced disease is the thymus. Preliminary experiments indicate that if the BALB thymus is grafted into thymectomized F_1 hybrids between the BALB and Strong A strains, the grafted thymus may become lymphomatous if the F_1 host is subjected to the combined action of x-rays and estrogenic hormone.

"Co-leukemogens" analogous to co-carcinogens have not been demonstrated. A co-leukemogen would be an agent which augments the action of a leukemogen, but is not in itself leukemia-inciting. For the DBA strain, estrogenic hormone may be a *co-leukemogen*, since in these mice there is no evidence that estrogenic hormone is leukemogenic. Large doses are not tolerated, but in doses that are at least mildly leukemogenic for the BALB and CBA strains, estrogenic hormone does not induce leukemia in DBA's. Its potentiating effect on methylcholanthrene leukemogenesis is very decisive (Chart 4, *f*), and there is a significant accelerating influence on x-radiation leukemogenesis. Since androgenic hormone opposes the leukemogenic action of x-rays (2, 7) and of estrogen (3), its effect on methylcholanthrene leukemogenesis is now being tested.

If any area of the body of C57BL mice was shielded during exposure to leukemogenic doses of x-rays, then leukemia was only infrequently induced, suggesting that nonirradiated tissue may in some way inactivate a humoral leukemogen emanating from the radiated portion of the body (6). Shielding of a portion of the body did not in BALB mice inhibit the acceleration of leukemogenic action resulting from the combined effects of x-rays and estrogen. This suggests that the estrogen may have prevented the inactivation and indeed enhanced the potency of the theoretical humoral agent.

Since the thymus was the primary locus of origin of leukemia even in BALB mice in which the thymus had been shielded during irradiation, the evidence points again toward a humoral factor which can be potentiated by estrogen, and which may actually implement neoplastic alteration by secondary effects in a thymus not radiated by x-rays (Chart 1, *e*).[4] This phenomenon did not occur in CBA mice where shielding of a part of the body apparently prevented the x-rays from operating in conjunction with exogenous estrogen to induce leukemia. Thymic radiation of estrogen-treated CBA mice did not induce thymic lymphosarcoma as in BALB mice.

Leukemogens may be defined only in terms of the biological material within which they may operate effectively to induce leukemia. Methyl-

[3] A. Kirschbaum, unpublished data.

[4] It has been calculated that the lead-shielded thymus receives approximately 1 roentgen per minute for each 100 r given to the remainder of the body.

cholanthrene induces leukemia in DBA but not CBA mice. On the other hand, ionizing radiations are probably universally leukemogenic for mice, but with varying strain susceptibilities.

Genetic factors determine the reaction to the combined effects of leukemogenic agents. In DBA mice methylcholanthrene augments the leukemogenic potency of x-rays and acts synergistically with estrogenic hormone, whereas in CBA mice this carcinogenic hydrocarbon exerts neither effect.

SUMMARY

1. Although susceptible to the leukemogenic action of x-rays or estrogenic hormone, BALB and CBA mice were relatively refractory to methylcholanthrene. X-rays and estrogenic hormone acted synergistically in accelerating the onset of induced leukemia. Methylcholanthrene did not act synergistically with either x-rays or estrogenic hormone in these strains.

2. In DBA mice lymphosarcoma and leukemia were induced by either methylcholanthrene or x-rays. Estrogenic hormone enhanced the leukemia-inciting potency of either the carcinogenic hydrocarbon or the ionizing radiations, although independent leukemogenic activity of estrogenic hormone in the dose used was not demonstrable in DBA's.

3. Genetic factors determine susceptibility to specific leukemogens acting either independently or in conjunction with others.

4. "Synergism," as used here, denotes increasing, by the simultaneous administration of leukemogens in relatively low doses, the incidence of lymphomas in the first 300–400 days of life beyond the sum of the incidences obtained by the independent action of the leukemogens.

5. The thymus was the most important locus for the synergistic effects of leukemogens (x-rays, methylcholanthrene, estrogen) in accelerating the onset of induced leukemia.

6. In BALB mice radiation restricted to the thymus accelerated the onset of thymic lymphosarcoma when given in conjunction with estrogen, whereas in CBA's thymic radiation was ineffective under the same conditions.

7. Thymic lymphosarcomas developed precociously in BALB mice after the whole body, except the thymus, was subjected to radiation and to relatively small doses (from the standpoint of leukemogenesis) of estrogenic hormone. Since thymectomy abolishes the leukemogenic activity of these agents in BALB mice, it is probable that the radiation effects on the thymus were secondary, or humoral, and were elicited by the action of estrogenic hormone.

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Resistance to Tumor Implantation Induced by Red Cell Stromata*

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INTRODUCTION

Although induction of resistance to transplantable tumors in mice by a prior inoculation of normal mouse cells was first demonstrated many years ago, there still remain several points which require investigation. A prior publication from this laboratory (1) reviewed the literature and reported experiments which showed that the genetic interrelationships among the materials used have an important influence upon the results obtained. Various degrees of resistance were observed in heterogeneous combinations of materials, but no resistance could be induced in an inbred strain of mouse against a tumor that arose in the same strain. This supports the concept that the degree of "foreignness" of the materials introduced into the host animal is a primary factor in the reaction. However, it has never been clear what this "foreignness" actually comprised or what it was in the cell which expressed this quality.

In experiments performed to gain further information regarding the nature of the "antigen" concerned in this type of "immunity,"[1] it was found that under the proper conditions, the power of a suspension of erythrocytes to induce immunity was comparable to that of defibrinated blood in equivalent doses (2). Thus, the plasma is apparently not concerned and, what is more important, the cell which serves as antigen does not need to have a nucleus. This seems to circumscribe and simplify the problems involved in the study of this type of immunity.

Our working hypothesis involved the idea that the antigenicity under investigation was an attribute of the cell surface and might depend upon a degree of organization above that of large molecules. With this in mind, cell suspensions were subjected to disintegration by three methods which were relatively gentle chemically and largely physical in nature—namely, osmotic laking with water, centrifugation while thawing from the frozen state, and brief exposure to high-pitched audible sound waves. It was assumed that, if the antigen were a molecular substance comparable to known antigens, it should survive one or more of these procedures and retain the power to induce a significant degree of immunity. However, the cells lost all power to induce resistance when they were broken up completely by either of the three methods of laking (2). It seemed reasonable to assume that the methods employed would produce in the cells chemical changes of only a minor degree which were not likely to be the basis for the major changes in the antigenicity observed. On the other hand, the physical changes in the cells were of a major degree. With each of the technics used, the cells were ruptured with sufficient violence to destroy almost all of the cell membranes; and, focusing upon that point, we were led to adopt, tentatively, an interpretation which was primarily physical rather than chemical.[2] It then became desirable to know what results would be obtained if cells were laked by methods which would preserve most, or all, of the cell membranes. In other words, we wished to learn whether red cell stromata would by themselves induce resistance against the implantation of a tumor. The following experiments will show that they do.

MATERIALS AND METHODS

The materials and methods used were the same, except for experimental variations to be described, as those used previously (2).

* This manuscript was prepared while the senior author was a summer investigator in the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

[1] To avoid more cumbersome terminology the term "immunity" will be used hereafter to refer to the host's resistance to the implantation of a tumor graft, and the term "antigen" will be used to indicate a substance capable of inducing such a state of immunity without reference to the presence or absence of demonstrable antibodies.

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[2] These terms are used in the sense that the arrangement of things is physical in nature and the substance of things is chemical in nature. In using this terminology we are aware that there is no sharp line between the physical and the chemical and that ultimately this distinction may become indistinct and no longer useful. However, the distinction seems to add clarity for the present.

All the hosts were strain BALB/cN (4), approximately 8 weeks old. Both sexes were used and distributed equally throughout the experiments.

The immunizing injection was material derived from normal strain DBA/2N (4) blood in all cases and represented pooled defibrinated blood from a small number (usually six to twelve) of donors which were sometimes young adults and sometimes retired breeders, according to the availability of animals, and of both sexes. No differences ascribable to the age or sex of the blood donors were detected. The immunizing dose was always the amount of material which was derived from, or equivalent to, 0.02 cc. of defibrinated blood.

The incubation period elapsing between the immunizing injection and the inoculation of the tumor graft was always 21 days.

The transplantable tumor inoculated was DBA sarcoma 49 (2). Although this tumor originated in strain DBA (Andervont's subline of line 2), it will grow progressively in approximately 90 per cent of strain BALB/cN mice. Transplantation was performed by inoculating 0.025 cc. of a 50 per cent tumor brei subcutaneously into the right flank of the host. The mice were then observed weekly for 4 weeks; the presence or absence of a tumor was noted; and, when present, the tumors were measured for the purpose of observing growth. The data are based upon the number of tumors present 3 weeks after implantation. This time interval was selected arbitrarily and is not essential for the conclusions.

Immune control mice were given the standard dose of 0.02 cc. of washed red cells prior to inoculation of the tumor. Six groups of twenty to 30 mice each were used, one group being concurrent with each of the experimental series.

Nonimmune control mice were given nothing prior to tumor inoculation. Six groups of ten each were suitably distributed through the experiments to afford assurance that the tumor continued to grow in a high percentage of normal strain BALB/cN mice.

Red cell stromata were prepared by three methods of laking, employing saline, citrate buffer solution, and hemolytic antiserum.

A "fragility" test with graded dilutions of saline showed that 0.3 per cent saline would produce complete hemolysis in 10 minutes and leave stromata in numbers representing approximately 80 per cent of the original cells. The pH of the suspension was 7.0. Stromata prepared in this way were brought to normal tonicity by adding sufficient dry salt to the suspension at the end of 10 minutes. They were then centrifuged out, washed once with normal saline, and resuspended in normal saline. Such a suspension was injected under the dorsal skin of mice in a dose equivalent to 0.02 cc. of defibrinated blood, allowance being made for the depletion in the number of cells during treatment. Five groups of hosts, with twenty to 30 mice in a group and a total of 129, were so immunized, and 21 days later they were inoculated with the tumor.

Stromata were also prepared by means of 0.02 M sodium citrate-citric acid buffer solutions at varied hydrogen ion concentrations. After 3 minutes' exposure to such solutions, laking was complete. The stromata were centrifuged out and resuspended in normal saline. The saline suspensions were then tested for their power to induce resistance in the manner described above. Stromata prepared at pH 7.0 were used in a group of 30 animals; stromata prepared at pH 5.5 were used in two groups of 30 animals each; and a preparation made at pH 4.0 was used in a group of 30 animals.

Hemolytic antiserum was obtained by injecting a Race III male rabbit[3] weighing 2,665 gm. with a 30 per cent suspension of washed strain DBA red cells. Doses of 1 cc. each were given intravenously on 3 successive days in each of 3 successive weeks. Two weeks after the last immunizing injection, sera from the immune rabbit and from a similar normal rabbit were harvested and prepared by the usual methods. The sera were not inactivated. When tested against a 5 per cent suspension of strain DBA red cells the antiserum (without additional complement) was hemolytic at a dilution of 1:32 and agglutinated at 1:256 after 30 minutes in a water bath at 37° C. Normal serum showed no hemolysis and weak agglutination at a dilution of 1:2. A dilution of 1:8 was selected for the experiment because, as will be described later, there was a strong contrast between the appearance of the cells in the two sera at this dilution. Red cells were suspended in normal serum and antiserum at that dilution (of serum) and incubated in the water bath for 30 minutes. Thereafter, they were tested for their power to induce resistance in the usual manner. Each preparation was tested in a group of twenty mice.

Stromata prepared with 0.3 per cent saline, as described before, were subjected to high-pitched audible sound vibrations at a frequency of 9 kc. for 90–120 seconds. Heating was prevented by circulating water at 2° C. The conditions were identical to those imposed upon intact red cells in previous work (2). In each of three experiments such vibrated stromata were injected into a group of 30 mice, and the mice were subsequently tested for

[3] An inbred race of white rabbits bred by Dr. T. B. Sawin at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

immunity to the tumor. Another similar suspension of stromata was exposed to sound vibrations under the same conditions except that the duration was 6 minutes. These stromata were used in the usual manner in a group of 40 mice.

RESULTS

In confirmation of previously reported results, 93 per cent of untreated strain BALB/cN mice had a growing transplant of the strain DBA/2N tumor at 3 weeks (six experiments totaling 60 hosts with 56 tumors); this was taken as the point of reference for results in "nonimmune" hosts. Among a total of 158 hosts "immunized" with a suspension of washed red cells in six experiments, there were 30, or 19 per cent, with tumors at 3 weeks. This also confirms previous results, with similar doses, and is taken as standard results for "immune" hosts under these conditions. Table 1 sets out these results and the various results obtained with other experimental conditions. For purposes of comparison, we have included the results of previously published experiments (2) in which the red cell suspension was laked by exposure to sound vibrations (9 kc. for 1½–2 min.) before injection into the animal.

In five experiments, stromata prepared with 0.3 per cent saline induced the same degree of resistance as would be expected with intact red cells at the same dosage. That is, there were 26 positive cases (20 per cent) in a total of 129 hosts. When examined under the phase contrast microscope these stromata were somewhat distorted but appeared to be reasonably good membranes morphologically. There was a very small number of membranes containing a few brown granules, and occasionally an intact red cell was seen. Previous investigations of the influence of dosage indicated that the small number of intact red cells present would not affect the results materially. To add strength to our opinion that the result was dependent upon the whole "equivalent" dose and not upon the few intact cells remaining, an ancillary experiment was done to see if 0.02-cc. doses of such stromata and intact red cells were additive in effect. The two doses (one of stromata and one of red cells) were given simultaneously to nineteen animals. Subsequent inoculation with the tumor yielded three positive cases (16 per cent). This is similar to a result of 15 per cent positive cases (46 hosts, seven tumors) obtained with a dose of 0.05 cc. of cells and indicates that the two doses were additive in effect.

Three experiments with stromata which had been subjected to treatment with sound vibrations similar to that previously reported for red cell suspensions yielded a result which was significantly different ($P < 0.001$) from that obtained with red cells. In a total of 89 hosts there were 46 successful transplants (52 per cent) at 3 weeks. This is exactly the percentage of takes previously observed after immunizing the hosts with 0.0031 cc. (equivalence in terms of defibrinated blood) of intact red cells and quite different from the 91 per cent of positive cases observed after using red cells exposed to the same sound vibrations. The difference in effect was accompanied by a difference in appearance of the two suspensions (red cells and stromata) after exposure to sound. Grossly, the red cell suspension was clear and "bright," whereas the stromata suspension was slightly opaque and had a "silky" luster. Microscopically, the suspension of vibrated red cells contained only a rare intact cell and the small amount of debris present was of small size (1 μ or less), whereas the suspension of vibrated stromata contained a few membranes representing most, or all, of a cell and many fragments about 2–4 μ in diameter.

TABLE 1

THE RELATIVE SUCCESS OF INOCULATING A STRAIN DBA SARCOMA INTO STRAIN BALB/c MICE AFTER A PRIOR INOCULATION OF RED CELLS OR STROMATA PREPARED UNDER VARYING CONDITIONS*

Antigen injected	Total mice	Total tumors	Per cent positive
None (nonimmune controls)	60	56	93
Washed red cells (immune controls)	158	30	19
Washed red cells (after 1½–2 min. sonic vibration—from [2])	55	50	91
Stromata prepared with			
0.3 per cent saline, pH 7.0	129	26	20
same—after 1½–2 min. sonic vibration	89	46	52
same—after 6 min. sonic vibration	40	38	95
0.02 M citrate buffer, pH 7.0	30	4	13
" " " pH 5.5	60	20	33
" " " pH 4.0	30	22	73
Stromata in rabbit antiserum	20	4	20
Cells in normal rabbit serum	20	1	5

See text for statistical analyses.

* All doses were equivalent to 0.02 cc. of blood. Incubation period 21 days. Tumors represent positive cases 3 weeks after tumor inoculation.

The suspension of stromata which had been exposed to sonic vibrations for 6 minutes was "bright" in gross appearance, like the red cell preparation, and microscopically it contained a negligible number of visible particles. Also, like the vibrated red cell suspension, it did not produce a detectable degree of resistance. In one experiment 40 "immunized" animals had 38 tumors (95 per cent) 3 weeks after inoculation. This does not differ from "nonimmune" controls.

Stromata prepared by means of 0.02 M citrate buffer induced a degree of resistance which varied

with the pH of the buffer used. Thirty animals immunized with stromata prepared at pH 7.0 had four tumors, or 13 per cent. That result is not significantly different from those obtained with either intact red cells or stromata prepared with saline. However, stromata prepared at pH 5.5 were less effective and yielded an intermediate result. Sixty animals were immunized with such stromata and, of these, twenty, or 33 per cent, were susceptible to the tumor. That result differs significantly from both nonimmune controls ($P < 0.001$) and immune controls ($P = 0.03$). It is also different from results obtained with stromata prepared with saline ($P = 0.05$) and stromata prepared with citrate buffer at pH 7.0 ($P = 0.04$). Stromata prepared at pH 4.0 were even less effective as an antigen. Of 30 animals immunized with such stromata, 22 (73 per cent) were susceptible to the tumor. Although this result is different ($P < 0.01$) from that in nonimmune controls, it also differs significantly from all other groups of immune animals. We did not not observe any difference in the microscopic appearance of wet mounts of these three preparations of stromata which correlated with the results, but the stromata prepared at pH 4.0 were unique in one respect; the brown granules which can be seen both inside and outside the membranes in such preparations were immobile after exposure to pH 4.0, whereas in all other preparations (including that with saline) they were dancing. Otherwise, these stromata all had the same approximately normal, though somewhat distorted, appearance as those prepared with saline.

Stromata prepared by means of hemolytic antiserum produced a result which was the same as that obtained with stromata prepared with saline and was not significantly different from that following the use of citrate buffer solution at pH 7.0. Of twenty hosts in this experiment, four, or 20 per cent, were susceptible to the tumor. Among the twenty additional control animals which were immunized with unlaked cells in normal rabbit serum there was one (5 per cent) positive case. These two results do not differ significantly ($P = 0.16$) between themselves or from immune controls ($P = 0.92$ and 0.13, respectively), but they are both different from nonimmune controls ($P < 0.001$). However, there was a difference in the appearance of the cells after treatment with the two sera which is of some interest. The suspension containing antiserum showed complete hemolysis and strong agglutination, while that containing normal serum showed no hemolysis and minimal agglutination. Under the phase contrast microscope wet preparations of these cells showed normal discoid appearance, "flicker" (3), very little agglutination, and only an occasional ghost in the normal serum, whereas in antiserum the cells were spherical, clumped, without "flicker," and only an occasional cell contained hemoglobin.

DISCUSSION AND CONCLUSIONS

A discussion touching upon all the work of others which might have some bearing upon these experiments would be unduly long. Discussions of such work can be found in reviews by Woglom (16), Spencer (15), and Snell *et al.* (13). Experiments with animals of unknown or heterogeneous constitution will not be mentioned, because they involve such a large complex of variables, many of which are unknown or uncontrollable, that we are unable to relate them to this work with certainty.

That special part of the field of tumor immunity which comprises the alteration of the susceptibility of animals to tumor implantation by pretreatment with nonliving materials has been reviewed by Snell *et al.* (13). Greatest interest attaches to the effect of the injection of lyophilized tissues, which has been studied by Snell, Kaliss, and their coworkers (10, 11, 13, 14). They have usually observed an enhancement of susceptibility to tumor implantation, but in some cases inhibition was observed, and the latter effect raises a question of the relationship of their phenomena to ours. However, at present, the differences between their experiments and ours seem more impressive than the similarities, for the following reasons. Firstly, the observations which they have reported include a qualitative change in the effect, i.e., enhancement in most cases and inhibition in some, whereas we have observed only inhibition or no effect, according to conditions. With variation of strains of mice in heterogeneous systems they obtained the qualitative reversal of effect, as well as the expected quantitative variations, whereas with variation of strains we obtain only quantitative variations of the same qualitative effect. Secondly, with variation of dosage they have observed a qualitative change in the effect, whereas we have seen only a quantitative effect of dosage. It must be remarked that their dose was expressed in terms of dry weight, while ours was expressed in volume. However, rough calculations based upon specific gravity and water content indicate that the seven dosage levels employed by us (i.e., 0.003–0.2 cc. equivalence of cells) and two higher levels employed by earlier workers (16) (0.3 and 0.5 cc. of defibrinated blood) would, if expressed in dry weight, range from well above the higher doses with which they obtained enhancement down to the lower doses with which they obtained inhibition; we always observed inhibition. Thirdly, their effect was ex-

pressed (14) as a difference in the size of the tumors which, in the case of inhibition, might be accompanied by an increase, decrease, or no change in the percentage of hosts susceptible to the tumor, whereas our effect was always seen as a decrease in the percentage of hosts susceptible to the tumor. Lastly, but in our opinion not least, their effect was observed after freezing, drying, and powdering the tissues, whereas the effectiveness of our "antigen" was destroyed by disintegration of the cell, and the disintegrated material gave rise to no enhancing effect detectable by our methods. One must conclude that the material with which they worked cannot at present be compared to the antigen which we are investigating. Furthermore, without prejudice to their data, we are of the opinion that thus far it is a little easier to associate their work with studies on "hypersusceptibility"—to borrow a term from the early literature (16)—especially since this is the effect more often observed, and to postpone a decision regarding its relationship to "induced resistance" with which this paper is concerned.

Regarding the induced resistance or immunity to transplantable tumors which can be produced by pretreatment with cells, Woglom (16) and Spencer (15) adopted the view (prior to the publication of [13]) that there was no satisfactory evidence that resistance could be induced by nonliving materials. The investigation most often referred to in this regard is that of Haaland (8), who said that cells "devitalized" by freezing and grinding were devoid of power to immunize against transplantable tumors. It now appears that such a method of killing cells would not only "devitalize" them but would also "disorganize" them, and the latter may be as important as the former. Formerly we accepted, tentatively, the then existing evidence that the power to induce resistance was a property of "intact, living cells." With later work (2), the emphasis shifted to the word "intact," and we began to suspect that architecture or organization (above the molecular level) might be an important determining factor. The results reported here seem to give encouragement for maintaining such a viewpoint on a working basis until more of the subtleties are known.

The data of Table 1 leave no doubt that a high degree of resistance against the implantation of a tumor can be induced by a prior injection of red cell stromata. Furthermore, the effectiveness of the stromata in inducing such resistance does not appear to be less than that of an equivalent dose of red cells. The aggregated data from experiments in which stromata were prepared at pH 7.0 show 34 tumors in 179 hosts, or 19 per cent positive; this is the same result as was obtained in 158 hosts immunized with washed red cells. Generalization beyond the bounds of these experiments may not be justifiable, but there is no apparent reason for believing that these results will turn out to be unique and limited to the conditions of these experiments. The principal effect of this observation is to contradict the notion that immunity to the implantation of tumors can be induced only by "living" cells (possible exceptions are discussed in [13]). Until some future date when the boundary between "living" and "dead" can be accurately defined—if it ever can be—it would be fruitless to argue this point at length. Nevertheless, it is the opinion of the authors that it would be going too far to call these stromata "living" in the ordinary meaning of that term and that this point has lost whatever significance it might have once had.

Whatever the nature of the antigen involved in this type of immunity is, the fact that it is not limited to the intact living cell seems to open new possibilities for further elucidation. Thus far we have found that, of six methods of laking reported, those technics which left few large fragments of the cell membrane ablated the power to induce resistance, whereas those technics which left many large fragments of the cell membrane did not. Furthermore, when treated with sound waves red cells quickly lose all their antigenic power (of this type), and this is accompanied by reduction to small particle size, whereas stromata similarly exposed are not completely broken up and do not lose all antigenic power. Only after exposure to sound waves for 3 or 4 times as long a period were stromata observed to be reduced to small particle size and to become nonantigenic.[4] It is this apparent correlation between the degree of disintegration of the material and the degree of destruction of antigenicity which leads us to adopt tentatively a physical viewpoint of the phenomena, although we recognize that other viewpoints are tenable.

Undoubtedly some minor chemical alterations at the molecular level of organization take place in some or all of the procedures, but it is difficult to believe that such form the basis for the observations. This viewpoint with respect to the effect of high-pitched audible sound is supported by the work of others. In immunologic studies of mammalian cells Henle, Henle, and Chambers (9) reported that sound vibrations at a frequency of 8.9 kc. did not

[4] A possible explanation of the difference in resistance to sound vibrations may lie in the way that energy would be released at the interface between materials of different density. In the case of stromata, saline is both inside and outside the membrane, whereas cytoplasm (etc.) is inside the intact cell membrane and saline is outside.

destroy the head specific, tail specific, or species specific antigens of spermatozoa of man, dog, rabbit, guinea pig, or bull. The time of exposure in their work varied from 7 minutes to 1 hour, and the temperature was "not higher than 20° C." The morphology of the head or tail was not destroyed by these treatments except to separate the head and tail, and this, according to their interpretation, exposed a fourth antigen which was brought to the surface at the break. This is in contrast to our observations but supporting rather than contradicting, because in their work more rigorous exposure to sound vibrations did not destroy the cells nor the antigens. With respect to bacterial antigens, Chambers and Flosdorf (5) reported that the Vi antigen of *Eberthella typhi* survived vibration at a similar frequency for 45 minutes at 15° C. Mudd *et al.* (12) noted that under the same conditions the antigen responsible for the phagocytosis of a strain of *Streptococcus hemolyticus* survived for 1 hour. Chambers and Weil (6) reported that it required 60–75 minutes of such vibration to render the "irritating substance" of pneumococci nonantigenic. Crystalline egg albumin was not distinguishably different immunologically from native protein after exposure to a similar frequency for 4 minutes at pH 7 according to Flosdorf and Chambers (7). Thus, the specific qualities of antigens may survive much more severe exposures to sound vibrations (and presumably whatever chemical changes would be expected to result from such exposure) than were present in our work. In the case of spermatozoa very severe exposures to sound did not disintegrate the parts and did not destroy their special antigenic properties. In the case of red cells, the cell membranes were broken up and their special antigenicity was ablated by mild exposure to sound. However, one cannot be certain that the second attribute depends upon the first.

Although structure appears to be important, more than simple morphologic integrity must be considered. The cell membranes which were antigenically effective in our work were distorted and, as stated, only approximately intact representatives of the cell surface. Contrariwise, the best appearing stromata with which we have worked had been exposed to 0.05 per cent formaldehyde for 15 minutes (unpublished experiments), and these were devoid of the power to induce resistance (twenty hosts, twenty tumors). Similarly, heating at 56° for 30 minutes produced stromata which had an appearance similar to potent ones (unpublished experiments) but they also were devoid of immunizing power (twenty hosts, twenty tumors). The deleterious effect of lowered pH was not accompanied by commensurate morphologic changes. Thus, deleterious chemical effects may or may not be accompanied by correlated morphologic effects. A similar (but obverse) point can be made regarding the cells treated with serum; here, there were marked differences in appearance of the cells in the two sera but no difference in their immunizing power.

The existing data suggest that a degree of organizational integrity is essential in the nature of the "antigen" under investigation, but this does not demand either a whole cell or an undistorted one. In addition, as would be expected, relatively gentle chemical changes can also effect the potency of the antigen, but some of these may not destroy the potency if sufficient organization remains. One may imagine that in part, at least, the "foreignness" of the antigen may depend upon not only what is present but also how it is arranged. After all, this may be only a restatement of the basic concept of stereochemistry with reference to a different level of biological organization, and the same difference may be all that distinguishes this concept from the well known concepts of Landsteiner and Pauling.

An alternative interpretation might be that the essential nature of the antigen does not depend upon a supramolecular aggregate but that the development of immunity after injection of the antigen depends upon such an aggregate. Thus, the finely divided antigen might be functionally ineffective because of increased lability, quick removal from the depot, or for some other reason. A firm choice between these alternatives can be made only after further experimentation. The fact remains that no immunity was observed following the injection of finely divided antigen.

SUMMARY

Previous observations that a prior inoculation of washed red cells would, under proper conditions, induce a high degree of resistance against a subsequent inoculation of tumor were confirmed.

It was further observed that red cell stromata were capable of inducing resistance which was not distinguishably different in degree from that induced by intact red cells. This result was attained with stromata prepared by means of 0.3 per cent saline, 0.02 M citrate buffer solution, or hemolytic rabbit antiserum at or near neutrality. However, lowering the hydrogen ion concentration of the laking fluid to pH 5.5 or pH 4.0 produced a corresponding lowering of the degree of induced resistance.

Exposure of stromata to high-pitched audible sound vibrations ablated their power to induce resistance, but it was necessary to expose stromata

for longer periods of time than was required to produce the same effect in red cells. Under the influence of sound vibrations, the degree of loss of antigenicity of red cells and stromata was correlated with the degree of morphologic disintegration.

There was no discernible morphologic change which could be correlated with the graded effect on antigenicity of lowering the pH. On the other hand, there was no correlated change in the antigenicity accompanying the morphologic changes produced by antiserum.

It is concluded that the antigen involved in induced resistance is not limited in occurrence to the intact living cell. This antigen is sensitive to chemical change but, more important for future work, it seems to depend upon some degree of organization (or possibly aggregation). The level of organization required appears at present to be somewhere above the molecular level but not so high as that of the cell. Tentatively, one may suspect that the arrangement of its constituent parts is critical in the nature of the antigen, and this has been adopted as a working hypothesis.

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Obesity and the Time of Appearance of Spontaneous Mammary Carcinoma in C3H Mice*

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The relationship of tumorigenesis to diet has been investigated by many workers in the last several years. The effects of overfeeding and underfeeding experimental animals have been examined, and, generally, animals on a restricted food intake show some delay in spontaneously occurring and experimentally induced neoplasia (2, 3, 8, 9). Likewise, experimental evidence indicates that diets high in fat tend to promote or enhance the formation of many types of tumors in mice (5, 7). In most of these experiments the comparison has been made between an animal on a restricted or modified diet and one on unrestricted food intake. From such experiments no definite conclusions as to the effect of obesity on tumor formation can be drawn, since in these experiments none of the animals could be justifiably termed obese.

It has been recently reported that mice surviving a single toxic dose of gold thioglucose[1] develop varying degrees of true obesity (4). Weights of 60–80 gm. in males and 50–60 gm. in females were observed. Autopsy findings and carcass analyses of such obese animals show that the greatest increase in weight is due to the marked increase in the amount of adipose tissue. The ad libitum food consumption of the mice becoming fat exceeded that of controls during the period of development and maintenance of obesity (10). We have undertaken, in this study, to determine the effect of such obesity on the rate of appearance of spontaneously developing mammary carcinoma in mice.

MATERIALS AND METHODS

The C3H mice employed in this study were obtained from the Department of Genetics, University of California, and are a pure line strain with a high incidence of spontaneous mammary tumors. They were obtained with known birth dates at about 4 weeks of age, and all were females kept in a virgin state throughout the entire experiment. The animals were housed six to eight per cage and were allowed free access to Purina Laboratory Chow and water throughout the entire experiment. At 2 or 3 months of age (average weight, 22 gm.) the animals were divided into two groups, and the animals in one group were given a toxic dose of gold thioglucose (10 mg.) intraperitoneally. Survivors of this LD_{50} dose of gold thioglucose were then ear-tagged, weighed, and set aside to be observed at intervals with the control group. About 80 per cent of the mice treated with gold thioglucose became obese, while the remaining injected mice did not. These mice were examined periodically for spontaneous mammary tumors. The study was therefore based on three groups of mice, the effectual totals being 66 controls, 78 gold-treated obese, and ten gold-treated nonobese mice. The time of onset of tumor appearance as well as the subsequent duration of life was determined. When an animal died, the tumor was excised and examined histologically. The experiment was discontinued when 50 per cent of the mice of each group showed tumors.

RESULTS

In Table 1 are included for each group the weights of the animals, the survival times after onset of tumor appearance, and the times of appearance of spontaneous tumors in the 50 per cent of the animals studied. Thus, the ages when definite mammary tumors appeared in 38 obese and 33 control mice are tabulated. It is seen that at about 295 days 50 per cent of the mice in the obese group had spontaneous tumors. By comparison, the 50 per cent incidence time for the control group was about 354 days, a difference of 59 days. The average weight of the obese mice was 54 gm., and the span of life after onset of tumors was 64 days. The

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average weight of the controls was 30 gm. and their survival time 80 days. The group of mice injected with the same amount of gold but not becoming obese averaged 32 gm. in weight at the median time of appearance of tumors, which was 374 days, or about the same as that for the uninjected mice.

Chart 1 is a graph plotting the age of obese and control mice at the time of appearance of tumors against the cumulative percentage of animals developing tumors. It is seen that at 295 days of age only 19 per cent of the controls were tumor-bearing, compared to 50 per cent of the obese mice.

DISCUSSION

In this experiment we have compared the appearance of spontaneous tumors in three groups of C3H mice: in those made obese by injection of gold thioglucose, in gold-treated but nonobese mice, and in control mice. The obese animals attained an average weight of 54 gm., whereas the controls averaged 30 gm. This significant increase in weight is primarily an increase in adipose tissue, as shown previously. The occurrence of spontaneous tumors in the obese animals is significantly earlier. The median time of tumor induction in the combined groups was 332 days. By this time, 48 obese and 23 nonobese had developed tumors, whereas 28 obese and 43 nonobese had not developed tumors. Application of the median test (6) to these data warrants the conclusion that the difference is significant at $P = 0.0014$.

The results which we have obtained are in agreement with the work of others who have

TABLE 1

APPEARANCE OF SPONTANEOUS MAMMARY TUMORS IN OBESE AND CONTROL C3H MICE

	Control (33 mice)			Obese (38 mice)			Nonobese injected (five mice)		
Animal no.	Age at tumor onset (days)	Survival after tumor onset (days)	Weight at tumor onset (gm.)	Age at tumor onset (days)	Survival after tumor onset (days)	Weight at tumor onset (gm.)	Age at tumor onset (days)	Survival after tumor onset (days)	Weight at tumor onset (gm.)
1	168	52	24	152	75	45	221	43	33
2	218	104	26	171	89	49	323	102	32
3	239	80	31	175	45	42	337	83	30
4	243	41	26	177	64	55	339	70	34
5	250	75	28	177	70	48	374	76	32
6	265	68	33	193	17	53			
7	265	67	28	196	149	55			
8	273	62	30	197	62	46			
9	276	63	29	202	98	51			
10	277	74	28	213	28	56			
11	281	117	34	221	55	43			
12	287	110	32	222	63	55			
13	290	126	30	227	57	54			
14	312	41	29	227	57	60			
15	319	88	33	237	103	52			
16	319	88	35	237	50	55			
17	322	117	29	238	48	56			
18	323	107	34	243	68	52			
19	325	79	30	245	49	55			
20	325	141	29	246	73	59			
21	325	125	27	248	88	58			
22	332	86	32	256	76	59			
23	332	42	28	256	63	58			
24	334	103	32	260	37	48			
25	337	102	29	265	60	56			
26	337	66	29	270	5	59			
27	339	99	33	272	30	54			
28	343	49	30	277	77	56			
29	347	30	30	277	53	55			
30	347	30	30	283	46	55			
31	347	49	29	284	46	54			
32	347	77	32	291	42	48			
33	354	69	34	291	41	54			
34				291	91	54			
35				291	118	59			
36				294	50	46			
37				294	98	57			
38				295	84	57			
Av.	303	80	30	242	64	54	319	75	32

shown that animals on a restricted intake develop a smaller number of tumors than do the controls, and with other experiments where an increase in tumors has been shown to occur in those animals which have consumed a diet with higher caloric intake. The age when tumors appeared in the virgin female controls is similar to that found by Andervont in his experiments with virgin mice of this strain (1).

The possibility of carcinogenesis by gold per se may be considered and thus account for the earlier

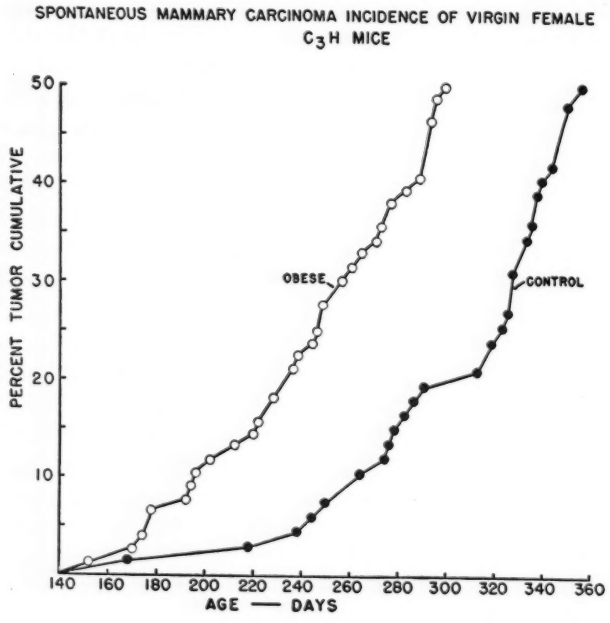

CHART 1

incidence of spontaneous tumors in the obese animals. However, the group of mice given the same amount of gold, but which did not become obese, showed no earlier incidence of tumors than was observed in the gold-treated obese animals. These mice had an average incidence of tumors similar to that of the controls which were not treated with gold and which were in the same weight range.

Experiments in progress with paired feeding of gold-treated potentially obese mice and nontreated controls show that both groups have approximately the same weight when given the same caloric intake, and the appearance of tumors is being noted in these animals.

SUMMARY

1. Virgin C3H female mice made obese experimentally with toxic doses of gold thioglucose show an earlier appearance of spontaneous mammary carcinomas than do control mice of normal weights.

2. The average time of appearance of spontaneous tumors in the obese mice was 246 days, compared to 303 days in the nonobese mice.

3. Animals injected with gold which did not become obese showed the same time of appearance of tumors as did the untreated mice.

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Quantitative Morphological Studies on the Livers and Liver Homogenates of Rats Fed 2-Methyl- or 3′-Methyl-4-dimethylaminoazobenzene

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Recent studies have suggested that the mitochondria in liver may undergo considerable variation in number under different physiological and pathological conditions. It has been found, for example, that the mass of mitochondrial material isolated from a hepatoma is much less than that obtained from normal liver (12, 13) and that the yield of mitochondrial material from the liver is greatly decreased after short periods of feeding carcinogenic azo dyes (10). The increases in certain mitochondrial-linked enzymes that have been observed in the livers of hyperthyroid and diabetic rats (19, 20) have indicated that the mitochondrial population of the liver may also undergo alterations under these conditions.

The development of a method for counting mitochondria in tissue homogenates (16) has permitted the direct determination of fluctuations in mitochondrial populations. In a preceding report (17), it was demonstrated that the number of mitochondria per unit weight of whole tissue was much lower in mouse hepatomas than in normal mouse liver, whereas the number of mitochondria per nucleus was approximately the same in the two tissues. In the experiments reported in the present paper the number of nuclei and mitochondria in liver homogenates was determined, and cytological and quantitative morphological observations were made on stained sections of the livers of rats fed the noncarcinogenic azo dye, 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB) or the highly active carcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB). In an accompanying paper (15), enzymatic and chemical determinations on the same livers will be reported.

MATERIALS AND METHODS

Adult male rats of the Holtzman strain, weighing 210–257 gm., were fed one of the four following diets: Purina Laboratory Chow, a basal semisynthetic diet,[1] the basal diet supplemented with 0.064 per cent 2-Me-DAB, or the basal diet supplemented with 0.064 per cent 3′-Me-DAB. The animals were allowed to eat ad libitum for 28 days. Although there were initially six rats in each of the four groups, two animals receiving 3′-Me-DAB died before the 28-day feeding period had elapsed, and two others (one on the basal diet and one receiving 2-Me-DAB) were sacrificed without analysis. Body weights were recorded before the animals were placed on the diets and at the termination of the feeding period.

The animals were killed with ether, and the livers were perfused through the portal vein with cold isotonic saline followed by cold 0.25 M sucrose. The livers were removed, weighed, and the median lobe (plus the left lateral lobe from the rats fed 3′-Me-DAB) was excised. Cross-sectional slices, 2 mm. thick, one proximal and one distal to the point of lobular attachment, were cut with a modified tissue slicer (5). These slices were divided into equal parts and placed into Helly's and into Baker's formol-calcium fixatives. The remainder of the lobe or lobes was squeezed through a tissue press, and weighed samples of the tissue mince were homogenized (7, 14) in 9 volumes of 0.25 M sucrose. All operations were carried out at 0–2°.

The tissues placed in Helly's fluid were fixed for 5 hours, washed overnight, and imbedded in paraffin. Sections (5 μ) cut from these liver slices were stained with hematoxylin and eosin and with eosin-azure buffered to pH 7.2 (3). The liver slices placed in formol-calcium were fixed for 24 hours, postchromated in 3 per cent potassium dichromate for 24 hours at 37° and imbedded in paraffin. Sections (3 μ) cut from this material were stained for mitochondria with anilin acid fuchsin-methyl green (3).

The mitochondria in the 0.25 M sucrose homogenates were counted in a Petroff-Hausser bacteria counting chamber using an AO-Spencer phase-contrast microscope equipped with 10 X oculars and a 1.8-mm. dark contrast oil immersion objective (15). Suitable dilutions were determined by the concentration of mitochondria in each homogenate. It was found that a 1:300 dilution of the liver homogenate from the rats maintained on both basal and chow diets was most satisfactory for accurate counting, while in the animals fed 2-Me-DAB the dilution was increased to 1:900, and in homogenates from livers of rats fed 3′-Me-DAB the dilution was decreased to 1-200. The final suspensions in each of the homogenates contained approximately 30–40 mitochondria per 10^{-3} c. mm. One or more

[1] The basal diet and the dyes (6; diet No. 3) used in this experiment were kindly supplied by Dr. J. A. Miller.

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samples from each of two dilutions was counted from each homogenate, with the combined count amounting to a total of over 370 mitochondria.

In counting nuclei, dilutions were prepared by mixing a 0.2-ml. aliquot of the homogenate with 7.8 ml. of 0.1 M citric acid containing 0.25 per cent methyl green. The suspensions were stirred and taken up in specially designed 15-ml. pipettes (11), which were sealed with broad rubber bands and rotated for at least 15 minutes on a Bryan-Garrey rotor (1) in order to obtain a uniform dispersion of the nuclei. The suspensions were introduced into a hemocytometer directly from the pipette, and the nuclei in all nine millimeter squares of the chamber were enumerated. The final estimate of the number of nuclei (Table 1) represented an average of the counts made from two or three dilutions from each homogenate.

TABLE 1

LIVER AND BODY WEIGHTS AT END OF 28-DAY FEEDING PERIOD

	Diet			
	Chow	Basal	2-Me-DAB	3′-Me-DAB
Liver weight (gm.)	11.0 (3)	10.2 (5)	10.6 (5)	7.8 (4)
Liver/body weight	0.046	0.041	0.056	0.041

Some difficulty was encountered in obtaining accurate counts of nuclei in homogenates prepared in 0.25 M sucrose, because in this medium the nuclei formed aggregates that could not be completely dispersed. It was therefore necessary to utilize duplicate liver homogenates prepared in 0.85 per cent NaCl, a medium in which the nuclei did not form clumps. The nuclear counts reported in Table 2 were obtained from saline homogenates. The average values obtained from the sucrose homogenates of the livers of the same rats were 19.9, 17.4, 15.8, and 35.3 million nuclei/ml for the chow, basal, 2-Me-DAB, and 3′-Me-DAB diets, respectively.

To determine the proportion of the whole liver that was occupied by parenchymal tissue and bile duct tissue, the method of Chalkley for the quantitative analysis of tissues (2) was applied to sections of the livers stained with hematoxylin and eosin. Five pointers were used in the ocular of the microscope, four of which were used for counting and the fifth for orientation. Hits on parenchymal cytoplasm, parenchymal nucleus, and bile duct cells were recorded separately, while hits on space and all other tissue elements were recorded as a fourth category ("unclassified"). For each twenty-five "throws" of the pointers (100 hits), the hits on the four categories were totaled separately on a differential blood counting tabulator, and the ratio of parenchymal cytoplasm to parenchymal nucleus was calculated. This value was calculated cumulatively, and when it remained constant within 3 per cent for six consecutive sets of 25 throws, the counting was discontinued. This evaluation was carried out on sections from both liver slices, so that the final estimate of cell volume was the mean value obtained from two different areas of the liver lobe.

Total nitrogen was determined on duplicate aliquots of the homogenates by the method of Ma and Zuazaga (4).

RESULTS

Liver and body weights.—The rats fed the basal diet gained an average of 15 gm., while the rats fed the diets containing 2-Me-DAB and 3′-Me-DAB lost an average of 42 and 38 gm., respectively, during the 28-day feeding period. At the end of the feeding period, the average liver weight of the animals fed 3′-Me-DAB was considerably lower than that of the livers of the rats fed the other three diets. The liver-body weight ratio of the 3′-Me-DAB-fed rats, however, was similar to that of the rats fed the chow or the basal diets (Table 1). Despite the fact that the rats fed 2-Me-DAB lost weight, the weight of the livers of these animals was maintained at a level similar to that of the control groups. This resulted in a much higher liver-body weight ratio than those of the rats of the other groups.

Total nitrogen.—The average amount of total nitrogen in the livers of the rats fed the basal diet or the 3′-Me-DAB was somewhat lower than the value obtained for the rats fed the chow diet. Feeding 2-Me-DAB resulted in a slightly increased amount of total liver nitrogen (Table 2).

Variations in mitochondria.—Table 2 summarizes the results of the counts of mitochondria in homogenates from all animals. In rats maintained on the chow diet, the number of liver mitochondria ranged from 89×10^9 to 134×10^9 per gram of liver with an average count of 118×10^9 (Table 2). Rats which were fed the basal diet had approximately the same number of liver mitochondria as those on the chow diet, the average being 120×10^9 per gram of liver. Ingestion of the diet containing 2-Me-DAB, however, resulted in a striking increase in the number of liver mitochondria. The average number of mitochondria in this group was 302×10^9 per gram of liver. The rats fed 3′-Me-DAB, with an average of 71×10^9 per gram showed a marked decrease in the number of mitochondria.

Variation in nuclear numbers.—Table 2 also presents the results of the counts of liver nuclei

from the four groups of rats. The number of nuclei in the livers of the rats fed the basal diet averaged 229×10^6 per gram of liver. This was similar to the value, 236×10^6 per gram, obtained for the rats fed the chow diet. In the case of the rats fed the 2-Me-DAB, the number of nuclei per gram of liver (192×10^6) was slightly less than the value for the rats fed the basal diet, while the livers from the rats fed 3′-Me-DAB contained greatly increased numbers of nuclei (519×10^6 per gram).

Calculations of the number of mitochondria per nucleus (M/N ratio) showed that this value was slightly higher in the livers of rats fed the basal diet as compared to those fed the chow diet (558 as compared to 467). Compared to these two values, the M/N ratio was much higher (1576) for the livers of rats fed 2-Me-DAB and much lower (115) for the livers of rats fed 3′-Me-DAB.

Results of quantitative morphological analysis.—By the Chalkley method of analysis, it was found that the parenchymal cells of the livers of the rats fed Purina chow represented 85 per cent of the volume of the whole organ, while the ratio of the volumes of parenchymal cytoplasm to nucleus was 15.1. As a result of feeding 3′-Me-DAB in the basal diet to the rats, the parenchymal cell volume fell to 55 per cent of the whole liver, and the ratio of parenchymal cytoplasmic volume to nuclear volume fell to 9.8 (Table 3). The cells of the bile duct epithelium in these livers had increased in number to such an extent that they represented approximately 16 per cent of the volume of the whole organ. The increase in the volume of the unclassified category appeared to be due to the increase in the amount of space and to the infiltration of histiocytes, granulocytes, and fibroblasts which accompanied the proliferation of the bile duct cells.

When the rats were fed the basal diet with or without 2-Me-DAB, the proportion of the volume of the whole liver represented by parenchymal tissue was increased by 5 per cent over that of the rats fed the chow diet. This was accompanied by an increase in the ratio of cytoplasmic to nuclear volume. The values obtained for the volume of whole liver occupied by bile duct tissue in the livers of rats maintained on the chow, basal, and basal plus 2-Me-DAB were considered only as an approximation, since accurate estimates of such low values could not be calculated on the basis of the number of counts made.

Histological and cytological observations.—The definite increase in the number of mitochondria in the parenchymal cells of the livers of the rats fed 2-Me-DAB has been reported previously by Price *et al.* (10) and was confirmed in the present experiment. In addition, the parenchymal cells seemed to be slightly larger than those of the rats fed the chow diet (Fig. 2; compare with Fig. 1), an impression which was confirmed by the Chalkley analysis which indicated an increase in parenchymal cytoplasmic volume in these livers.

While the normal architecture of the liver was not disturbed after feeding 2-Me-DAB, some alterations were observed in the cells adjacent to the central veins. In these cells there were hyaline inclusions of varying size and shape which did not stain with any of the stains used; furthermore, the mitochondria were smaller in size and more closely packed together than in cells in other areas of the liver. Another definite and striking change observed in these cells was an almost complete absence of any material that would take a basic stain. Thus, in many cells even the nucleoli failed to stain with basic dyes (Fig. 5).

The general progression of changes taking place in the livers of rats fed 3′-Me-DAB over a long period of time has been described by Price *et al.* (9). The present brief report concerns only those

TABLE 2

TOTAL NITROGEN, NUMBER OF MITOCHONDRIA, AND NUCLEAR COUNTS IN RAT LIVERS AT END OF 28-DAY PERIOD

The values given in the table for nitrogen and for number of nuclei and mitochondria are for 1 gram of perfused liver

Diet	Nitrogen Mg	No. of Mitochondria $\times 10^{-9}$	No. of nuclei $\times 10^{-6}$	Mitochondria /nuclei
Chow	30.1	130		
	26.9	123		
	27.2	125		
	28.8	134	224	600
	26.9	106	240	443
	26.4	89	245	365
Av.	27.7	118	236	467
Basal	19.5	109		
	23.3	117		
	28.1	137	284	480
	22.1	116	175	660
	23.6	123	227	533
Av.	23.3	120	229	558
2-Me-DAB	30.2	282		
	25.6	329		
	25.0	288	222	1,297
	27.9	257	163	1,579
	32.2	352	190	1,853
Av.	28.2	302	192	1,576
3′-Me-DAB	20.3	62		
	23.0	84		
	23.7	64	642	99
	22.0	73	557	130
Av.	22.3	71	599	115

changes occurring after a 28-day dye-feeding period.

Feeding 3′-Me-DAB to rats resulted in a striking proliferation of bile duct epithelial cells. The bile duct cells, together with granulocytes, lymphocytes, histiocytes, and fibroblasts, tended to be concentrated along the spaces separating the lobules, but infiltration of the lobule up to the central veins frequently occurred. The extent of the proliferation varied among the individual rats and also varied among the slices taken from different areas in the same liver. In the liver of one rat there was only moderately extensive proliferation of the bile duct epithelium and little variability in the size of the parenchymal cells. The mitochondrial count from the homogenate of this liver was higher than that of the livers of the other rats (84×10^9 per gram, as compared to an average of 67×10^9 per gram). The variation in the volume of bile duct epithelium and parenchymal cells in different areas of the same liver is illustrated by the high standard deviation from the mean of the volumes of these components (Table 3), as compared to the low standard deviation of the parenchymal cell volume in the livers of rats fed the other diets.

In the livers of the few rats used in this study, it was rare to find the bile duct cells completely replacing the parenchymal cells as a result of feeding 3′-Me-DAB. In areas where the bile duct epithelium had extensively infiltrated the lobule, the hepatic cells had greatly reduced cytoplasmic volume, and this remaining cytoplasm was not infrequently vacuolated. The mitochondria in these cells were usually clustered around the nucleus and appeared to be reduced in number. There was considerable variation in the size of the parenchymal cells in general, since cytoplasmic and nuclear hypertrophy were commonly present (Figs. 3 and 4). The cytoplasm of most of the parenchymal cells was filled with large vacuoles, which hindered attempts to estimate with accuracy the relative mitochondrial numbers. The impression was gained, however, that, as the cytoplasmic volume of the cells decreased, the number of mitochondria also decreased, and it appeared that some of the hypertrophied cells actually had an increased number of mitochondria.

Another phenomenon worthy of note in the livers of the rats fed 3′-Me-DAB was the extreme attenuation of the chromosomes in the dividing parenchymal cells. The discreteness of the chromosomes was so striking that splitting of the chromosomes at metaphase was readily observed in tissue sections stained with hematoxylin and eosin (Fig. 6).

TABLE 3

QUANTITATIVE MORPHOLOGICAL ANALYSIS OF LIVER SECTIONS

The open figures represent mean values with the standard deviation. Figures in parenthesis represent number of tissue sections analyzed.

Diet	Parenchyma; per cent of whole liver	Bile duct tissue; per cent of whole liver	Unclassified; per cent of whole liver	Parenchymal cytoplasm/ Parenchymal nucleus
Chow	85±1.4 (13)	0.16 (13)	15.5±2.7 (13)	15.1±2.3 (13)
Basal	90±1.8 (10)	0.04 (10)	9.6±1.7 (10)	20.7±3.2 (10)
2-Me-DAB	91±2.5 (10)	0.06 (10)	9.5±1.7 (10)	24.2±1.7 (10)
3′-Me-DAB	55±8.9 (8)	15.7±5.9 (8)	29.2±4.8 (8)	9.8±3.6 (8)

DISCUSSION

The variation that has been observed in the amount of mitochondrial material and in the activity of certain mitochondria-linked enzymes in the livers of rats fed aminoazo dyes has been attributed to the changes in the number of mitochondria (8, 9, 10). The results of the present study confirm and extend these findings, since a 150 per cent increase in the number of mitochondria was found in the livers of rats fed the noncarcinogenic dye, 2-Me-DAB, while feeding 3′-Me-DAB resulted in a 41 per cent decrease in the number of liver mitochondria.

The increase in the number of mitochondria in the livers of the rats fed 2-Me-DAB was accomplished without a concomitant increase in the total number of cells in the liver (as estimated by the number of nuclei present[2]), and, despite the fact that the rats themselves lost weight, the weight of the liver was maintained on a level with that of the rats fed the basal diet. No record was kept of the food intake by these animals, and data are therefore not available to indicate whether this loss in body weight was due to decrease in food

[2] The authors are aware that the number of nuclei in a liver homogenate does not equal the number of cells present in the intact organ. The calculations of St. Aubin and Bucher (18) show that in the adult rats used in their experiments, binucleate cells constitute approximately 30 per cent of the total number of liver cells.

consumption. The livers of these animals, however, did not lose weight and even showed a slight but probably not significant increase in total nitrogen. The increase in mitochondrial nitrogen (15) was significant and seemed to indicate an actual synthesis of these organelles.

The decrease in mitochondrial numbers in the livers of rats fed 3′-Me-DAB was not easily interpreted because of the extensive proliferation of the bile duct epithelium and the wide variation in the size of the parenchymal cells. The presence of greatly increased numbers of bile duct cells which contained fewer mitochondria than the parenchymal cells might have accounted for the decrease in the M/N ratio, but analysis of the data indicated that the hepatic cells on the average contained fewer mitochondria.

The livers of the rats fed the basal diet had an average of 228×10^6 nuclei per gram, while the livers of those fed 3′-Me-DAB had an average of 600×10^6 nuclei per gram; thus, the number of nuclei per whole liver was $2{,}335 \times 10^6$ and $4{,}680 \times 10^6$ for the two diets, respectively. The Chalkley analysis indicated that the "basal livers" consisted of 90 per cent parenchyma, while the "3′-Me-DAB livers" contained only 55 per cent parenchyma by volume. Taking into consideration the respective liver weights and assuming that changes in weight reflected similar changes in volume (the "3′-Me-DAB livers" were grossly much smaller than the "basal livers"), the "basal livers" contained 9.2 cc. of parenchyma, and the "3′-Me-DAB livers" contained 4.3 cc. of parenchyma. But the ratio of cytoplasm to nucleus in the "basal livers" was 20.7 and in the "3′-Me-DAB livers" the C/N ratio was 9.8. Thus, although the volume of parenchyma in the "3′-Me-DAB livers" was only half that of the "basal livers," the number of parenchymal liver cells as represented by nuclear volume remained comparable to that of the "basal livers." The decrease in volume was therefore mainly a cytoplasmic rather than a nuclear change.

It appeared, then, that the parenchymal cells in the rats fed 3′-Me-DAB contained considerably fewer mitochondria than the liver cells of the rats fed the basal diet. The values for parenchymal volume, however, represent an average, and examination of the liver sections stained for mitochondria showed that some of the cells contained at least as many mitochondria as the cells in the livers of the rats fed the basal diet, while in many of the smaller parenchymal cells the number of mitochondria seemed to be decreased. Whether these findings relate to the process of carcinogenesis still remains unclear.

SUMMARY

1. Four groups of male rats of the Holtzman strain were fed Purina chow, a basal semi-synthetic diet, the basal diet supplemented with 0.064 per cent 2-Me-DAB, and the basal diet plus 0.064 per cent 3′-Me-DAB, respectively. The animals were allowed to eat ad libitum and were killed after 28 days of feeding. The nuclei and mitochondria present in homogenates of the livers of these animals were counted. Stained tissue sections of the same livers were analyzed by the method of Chalkley (2) for the percentage of the liver volume occupied by parenchymal cytoplasm and nucleus, and bile duct tissue.

2. Feeding 2-Me-DAB resulted in a striking increase in the number of mitochondria in the liver, 302×10^9 per gram, as compared to 118×10^9 and 120×10^9 per gram liver for rats fed the chow or the basal diet. The rats fed 3′-Me-DAB, with an average of 71×10^9 per gram, showed a marked decrease in the number of mitochondria.

3. The average number of nuclei per gram (599×10^6) in the livers of rats fed 3′-Me-DAB was much greater than that found in the livers of the rats fed the chow or basal diets (236×10^6 and 229×10^6 per gram, respectively). There were fewer nuclei per gram (192×10^6) in the livers of rats fed 2-Me-DAB than were found in the livers of the control animals.

4. Quantitative analysis of the tissue sections showed that, on an average, the livers of the rats fed 3′-Me-DAB contained only 55 per cent of parenchymal tissue, while the livers of rats fed chow, basal, or basal plus 2-Me-DAB contained an average of 85, 90, and 91 per cent of parenchymal tissue, respectively. Further analysis of the data showed that the decrease in the volume of parenchymal tissue in the livers of rats fed 3′-Me-DAB was the result of a decrease in volume of parenchymal cytoplasm, while the volume occupied by the nuclei remained comparable to that of the livers of rats fed the basal diet. It was concluded that the number of mitochondria in the average parenchymal cell of the livers of rats fed 3′-Me-DAB must be considerably reduced in comparison with the parenchymal cells of rats fed the basal diet.

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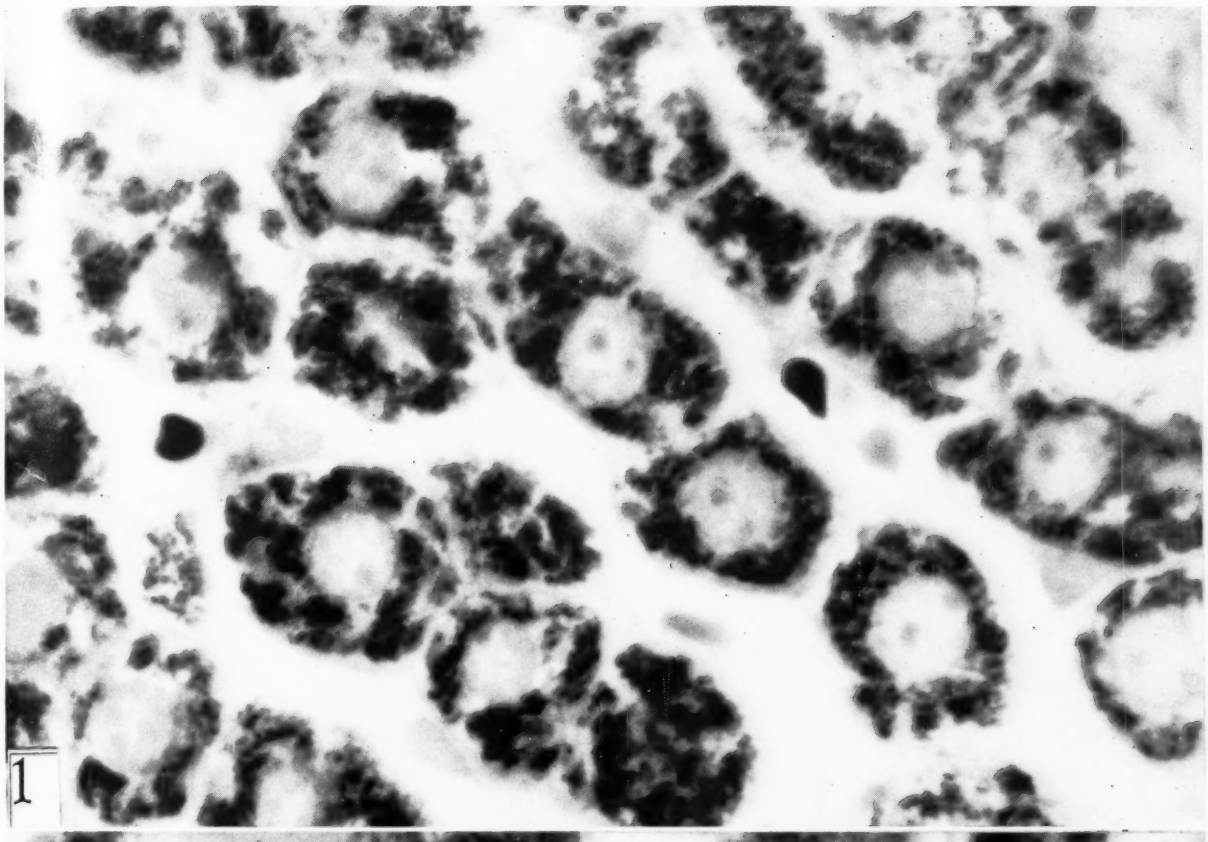
Fig. 1.—Mitochondria in parenchymal cells of a rat fed the basal diet. Aniline acid fuchsin-methyl green stain. ×1,600.

Fig. 2.—Mitochondria in parenchymal cells of a rat fed 2-Me-DAB. Note the increase in cytoplasmic volume. Aniline acid fuchsin-methyl green stain. ×1,600.



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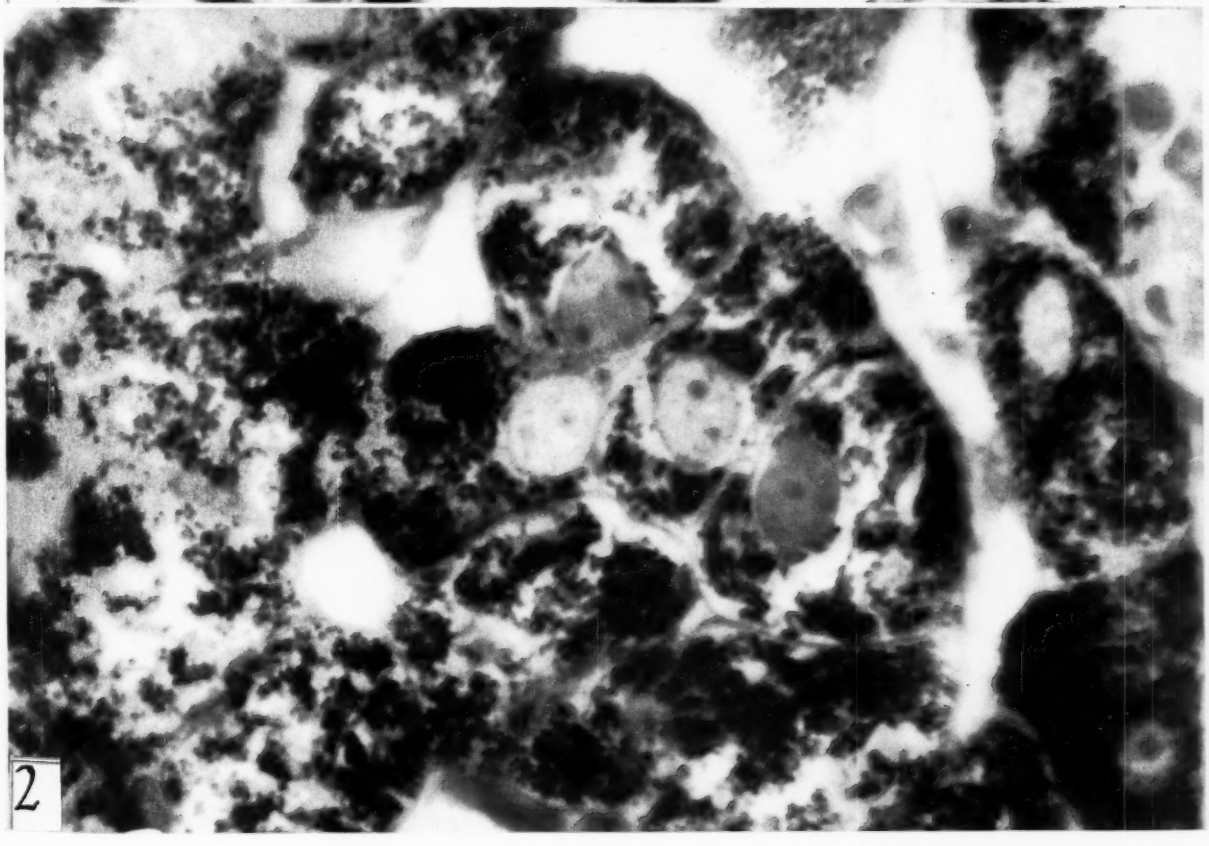

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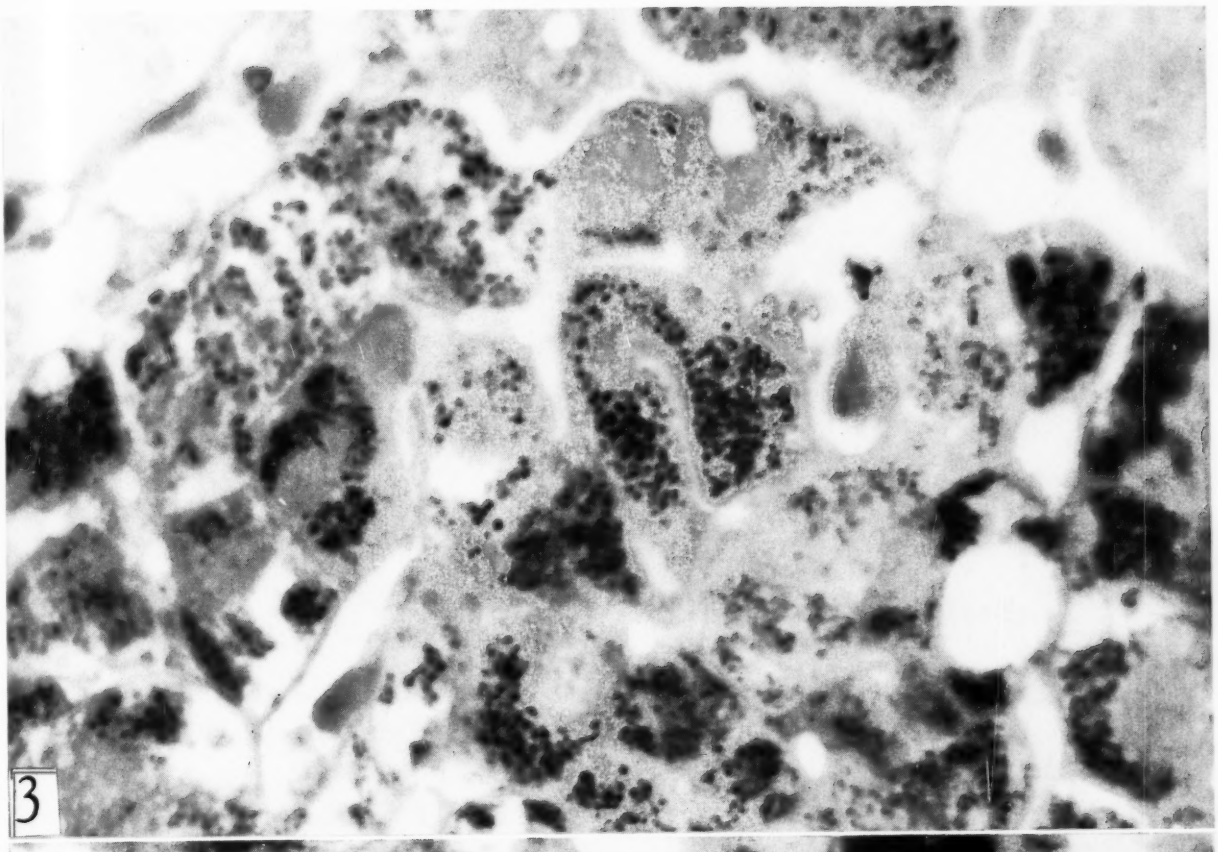
FIGS. 3 and 4.—Mitochondria in parenchymal cells of a rat fed 3′-Me-DAB. Note the variation in amount of cytoplasm and in the number and size of the mitochondria. Aniline acid fuchsin-methyl green stain. ×1,600.



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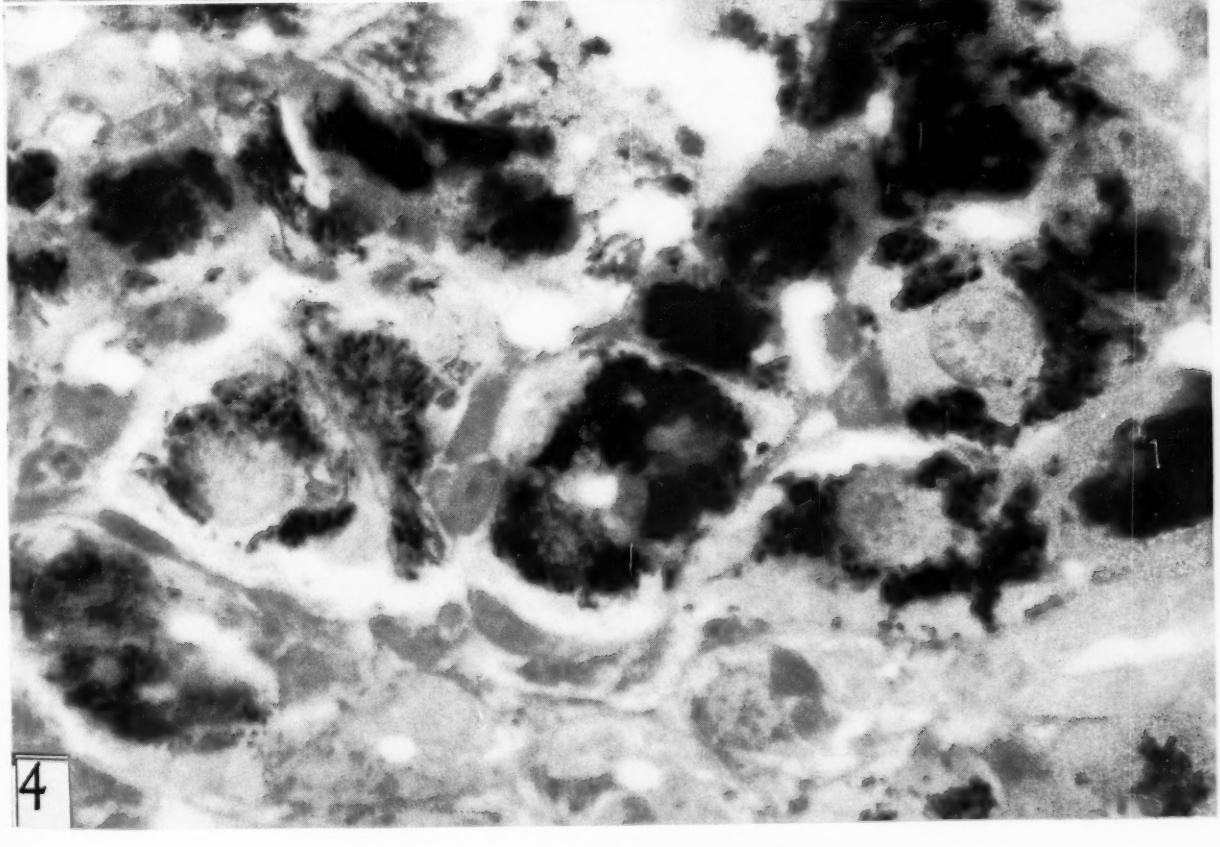

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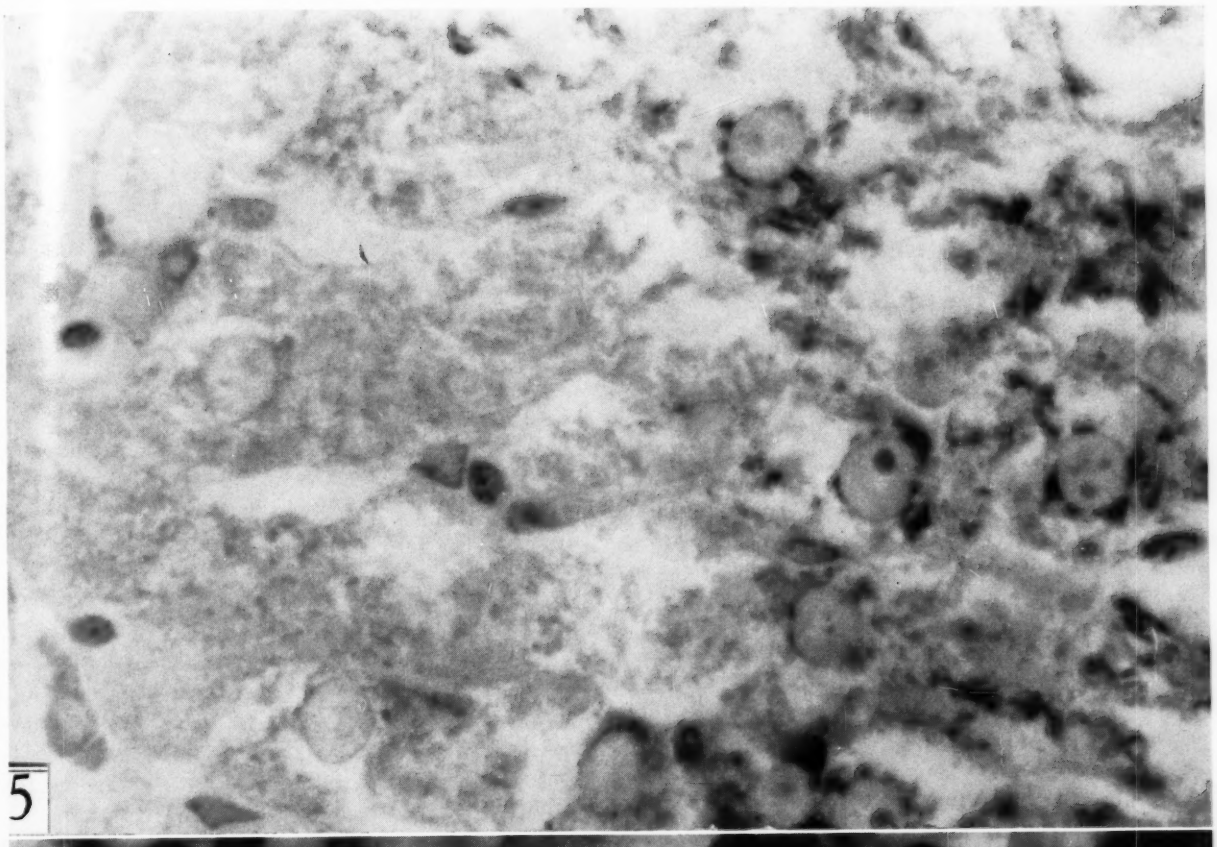
FIG. 5.—Cells adjacent to central vein in liver of rat fed 2-Me-DAB, showing absence of cytoplasmic and nucleolar basophilia. That this phenomenon is not a staining artifact is evidenced by the staining reaction of the small cells in the liver sinusoids. Lumen of central vein in lower left hand corner. Eosin-azure stain. ×1,300.

FIG. 6.—To illustrate the attenuated state of the chromosomes in the liver parenchymal cells of a rat fed 3′-Me-DAB. Hematoxylin-eosin stain. ×1,900.



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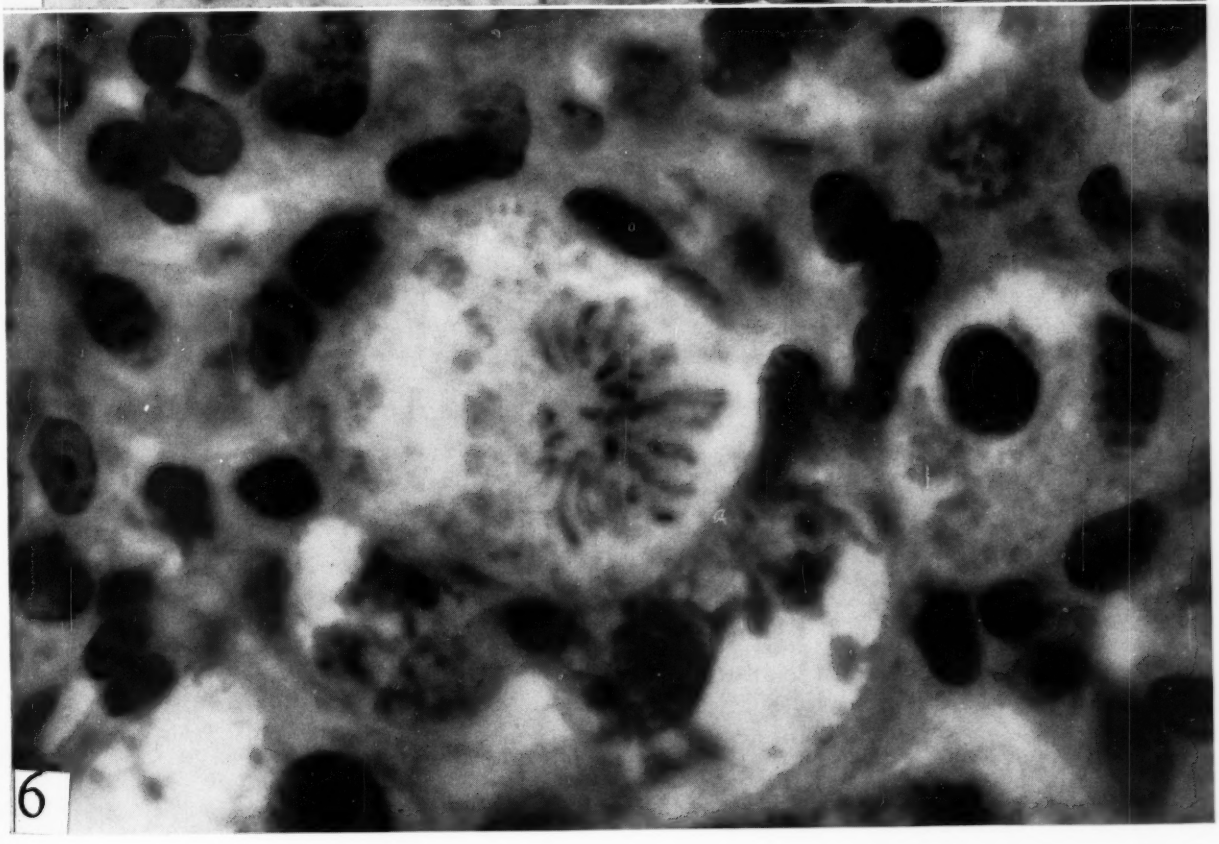

6

Enzymatic and Chemical Studies on the Livers and Liver Mitochondria of Rats Fed 2-Methyl- or 3′-Methyl-4-dimethylaminoazobenzene

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Price *et al.* (12) and Potter *et al.* (7) observed that the livers of rats fed the noncarcinogenic azo dye, 2-Me-DAB[1], contained increased amounts of isolated mitochondrial material and of a mitochondrial enzyme complex, the succinoxidase system, while the livers of rats fed the highly active carcinogen, 3′-Me-DAB[1], contained less mitochondrial material and lower succinoxidase activity than did control livers. In the preceding paper (24), we have demonstrated by means of direct counting procedures a large increase in the number of mitochondria in the livers of rats fed 2-Me-DAB and a decreased number of mitochondria in the livers of rats fed 3′-Me-DAB. The present paper reports the results of enzymatic and chemical studies on the same livers that were used in the preceding paper for the mitochondrial counts. In addition to measurements of succinoxidase activity, the activity of a number of other enzymes which are normally associated with liver mitochondria was also determined. The enzymes studied include the fatty acid oxidation system (octanoxidase), uricase, and DNase[1] and RNase[1]. DPN-cytochrome c reductase, an enzyme that has been found to be associated with both mitochondria and submicroscopic particles in normal liver (2, 3) was also studied, as were the concentrations of the nucleic acids in these livers.

MATERIALS AND METHODS

The rats used in the present experiments were the same as those described in the preceding paper (24) and were maintained for 28 days on either a commercial chow diet, a semisynthetic basal diet, or the latter supplemented with 2-Me-DAB or 3′-Me-DAB. On the day of each experiment analyses were carried out on either the livers of three rats on the chow diet or the livers of one basal rat, one 2-Me-DAB rat, and one 3′-Me-DAB rat. The details of the dietary regimes and the preparation of the liver homogenates are given in the preceding paper (24).

The liver homogenates in 0.25 M sucrose were fractionated at 0° by differential centrifugation, as described previously (16), to isolate the mitochondrial fraction. The nuclear fraction and the supernatant that remained after the isolation of the mitochondria were discarded.

The number of nuclei and mitochondria in the homogenates and of mitochondria in the mitochondrial fraction was determined by counting procedures that have been described (23). The values given in Table 1 for the number of nuclei and mitochondria in the homogenates were reported in the preceding paper (24).

Total nitrogen was determined by the method of Ma and Zuazaga (6), and DNA and PNA were determined by means of the diphenylamine and orcinol reactions on hot trichloroacetic acid extracts of the livers (13).

Each of the enzymes studied in this paper was determined under conditions in which the activity was directly proportional to the amount of tissue added and the course of the reactions obeyed zero order kinetics. Each homogenate or mitochondrial fraction was assayed at 2 tissue levels and, with one exception, all assays were made as soon as the preparation of the mitochondrial fractions had been completed. The DNase and RNase assays were made on the day following the mitochondrial separation, since it was found that the activities of these enzymes were very little altered during storage at 0° for this period of time. Succinoxidase and octanoxidase activities were obtained from measurements of oxygen uptake at 38° (16, 21). DPN-cytochrome c reductase activity was determined spectrophotometrically from the rate of reduction of cytochrome c (4). Uricase activity was measured by following the destruction of uric acid spectrophotometrically (18). DNase and RNase activities were obtained from spectrophotometric measurements of the rate of formation of perchloric acid soluble fragments from DNA and RNA (19).

RESULTS

The results of the enzymatic assays, the chemical determinations, and the nuclear and mitochondrial counts are presented in Table 1.

Basal and chow diets.—The results in Table 1 show that the basal diet itself produces pronounced alterations in the liver. Although the numbers of mitochondria and nuclei were not appreciably different in the basal and in the chow

[1] The following abbreviations will be used in this paper: 2-Me-DAB = 2-methyl-4-dimethylaminoazobenzene; 3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene; DNase = desoxyribonuclease; RNase = ribonuclease; DPN = diphosphopyridine nucleotide; DNA = desoxyribonucleic acid; RNA = ribonucleic acid.

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livers, the amount of mitochondrial material, as measured by nitrogen determinations, was considerably less in the livers of the rats on the basal diet, and the activity of all enzymes studied, with one exception, was reduced in the livers on this diet. The DNase activity of the basal livers was greater than that of the livers obtained from the rats on the chow diet.

2-Me-DAB diet.—The enormous increase in the number of mitochondria that was observed in the livers of rats fed 2-Me-DAB ([24], Table 1) was accompanied by an increase in the total nitrogen basophilia observed cytologically in some areas of the 2-Me-DAB livers (24). The basophilia of the livers appeared to be decreased to a greater extent, however, than the PNA figures would indicate.

3′-Me-DAB diet.—The livers of the rats fed 3′-Me-DAB were characterized by decreased numbers of mitochondria, greatly increased numbers of nuclei, and by a decreased amount of parenchymal cytoplasm ([24], Table 1). The decrease in the latter, which was about 40 per cent, was of sufficient magnitude to account for the decreased

TABLE 1

ENZYMATIC ACTIVITIES AND CHEMICAL COMPOSITION OF THE HOMOGENATES AND MITOCHONDRIAL FRACTIONS OBTAINED FROM THE LIVERS OF RATS MAINTAINED ON VARIOUS DIETS

All values reported are for 100 mg. of perfused liver or its equivalent. The number of determinations are noted in parentheses.

	Chow diet		Basal diet		2-Me-DAB +basal diet		3-Me-DAB +basal diet	
Measurement	Homogenate	Mitochondrial fraction	Homogenate	Mitochondrial fraction	Homogenate	Mitochondrial fraction	Homogenate	Mitochondrial fraction
Total nitrogen (Mg.)	2.77 (6) ±0.11	0.666 (6) ±0.088	2.33 (5) ±0.15	0.538 (5) ±0.044	2.82 (5) ±0.24	0.914 (5) ±0.135	2.23 (4) ±0.11	0.288 (4) ±0.058
DNA phosphorus (μg.)			19.4 (2) ±1.2		17.0 (2) ±1.4		56.8 (2) ±2.5	
PNA " (")			52.4 (2) ±3.6	8.7 (2) ±0	49.5 (2) ±1.8	6.0 (2) ±1.6	53.5 (2) ±3.0	3.8 (2) ±0.5
Nuclei×10^{-6}	23.6 (3) ±0.8		22.9 (3) ±3.8		19.2 (3) ±1.4		59.9 (2) ±4.3	
Mitochondria×10^{-9}	11.8 (6) ±1.4	8.07 (3) ±0.3	12.0 (5) ±0.8	9.35 (5) ±1.7	30.2 (5) ±3.1	24.0 (5) ±2.6	7.08 (4) ±0.8	4.83 (4) ±0.5
Succinoxidase activity $mm.^3O_2$/10 min.	449 (6) ±72	278 (6) ±66	311 (4) ±39	198 (4) ±14	664 (4) ±97	474 (4) ±60	247 (3) ±29	132 (3) ±16
Octanoxidase activity, $mm.^3O_2$/10 min.		34.7 (3) ±2.2		26.5 (3) ±1.5		27.0 (3) ±4.1		6.1 (2) ±2.1
DPN cytochrome c reductase activity, μM cytochrome c/min.			4.40 (3) ±0.12	1.15 (3) ±0.14	6.54 (3) ±1.04	2.01 (3) ±0.21	2.50 (2) ±0.52	0.57 (2) ±0.23
Uricase, μM uric acid destroyed/min.	.097 (3) ±.002	.044 (3) ±.001	.069 (1)	.042 (1)	.076 (1)	.038 (1)	.044 (1)	.022 (1)
DNase, E_{260}/30 min.	5.99 (3) ±0.18	3.57 (3) ±0.17	7.61 (2) ±0.75	4.60 (2) ±0.36	7.25 (2) ±0.65	4.95 (2) ±1.00	24.0 (3) ±1.1	9.27 (3) ±0.82
RNase, E_{260}/30 min.	47.9 (3) ±3.1	28.9 (3) ±0.9	35.6 (2) ±0.6	17.6 (2) ±2.1	26.5 (2) ±4.2	18.1 (2) ±5.0	91.6 (2) ±3.8	20.1 (2) ±1.3

content of the mitochondrial fraction and in the succinoxidase and DPN-cytochrome c reductase activities of these livers. The increase in the succinoxidase activity of the 2-Me-DAB livers was almost as great as the increase in mitochondrial numbers, while the increase in mitochondrial nitrogen and reductase activity were much less. The activity of the other mitochondrial enzymes studied was not increased in the 2-Me-DAB livers (Table 1). The octanoxidase, uricase, DNase and RNase activities of the 2-Me-DAB livers were about the same as or slightly less than these activities in the basal livers. The amounts of DNA and PNA were lower in the 2-Me-DAB livers than in the basal livers, and the decreased DNA content was correlated with a decreased number of nuclei in the 2-Me-DAB livers, while the decreased amount of PNA in these livers was related to the decreased number of mitochondria (41 per cent less than basal value), the decreased nitrogen content of the mitochondrial fraction (46 per cent less), and the decreased DPN cytochrome c reductase and uricase activities (43 and 36 per cent less than basal values, respectively) of these livers. The decreased succinoxidase activity (21 per cent) and octanoxidase activity (77 per cent) of the 3′-Me-DAB livers could not be explained on this basis nor could the enormous increases in the DNase and RNase activities observed. The latter amounted to increases of 150 to 200 per cent and were closely correlated with increases of similar magnitude in the number of nuclei and in the amount of DNA in these livers (Table 1). Only a small proportion of the DNase (39 per cent) and RNase (22 per cent) activities of the 3′-Me-DAB livers was associated with the mitochondrial fraction, whereas

in the livers of the rats on the other three diets, 60–68 per cent of the DNase and 50–68 per cent of the RNase activities was recovered in the mitochondrial fraction. In one experiment not reported here, in which the DNase activity of all the fractions of a 3′-Me-DAB liver was determined, it was found that the activity was distributed evenly between the nuclear, mitochondrial, and supernatant fractions. It appears possible that the high DNase and RNase activities of the 3′-Me-DAB livers was due to the presence in these livers of nonparenchymal cells in which these enzymes were not associated with mitochondria.

DISCUSSION

The results of the present investigations ([24] and Table 1) confirm and extend the results of Price *et al.* (7) and Potter *et al.* (12). The results show that increased numbers of mitochondria were present in the livers of rats fed the noncarcinogenic dye, 2-Me-DAB, and that this increase occurred without any increase in the cellularity of the liver. The results in Table 1 show further, however, that the increased numbers of mitochondria in these livers could not be considered to have arisen by a simple process of multiplication, since the properties of the 2-Me-DAB mitochondria did not quantitatively duplicate those of the control mitochondria (Table 1). The duplication of mitochondria could of course be considered to involve division of the mitochondria followed by the synthesis of the mitochondrial constituents at varying rates until the quantitative composition of the daughter mitochondria equalled that of the original mitochondrion. Such a hypothesis would satisfactorily account for the fact that the increased succinoxidase activity of the 2-Me-DAB livers was almost the same as the increased number of mitochondria in these livers, while the increased total nitrogen content and cytochrome c reductase activity of the 2-Me-DAB livers was not so great as the increase in mitochondrial numbers, and the uricase, octanoxidase, and nuclease activities of these livers did not increase at all. The possibility should be considered, however, that the increased succinoxidase and cytochrome c reductase activities observed in the 2-Me-DAB livers did not represent actual increases in the total amounts of these enzymes but rather increases in their measured activity occasioned by the breakdown of the normal liver mitochondria into smaller sub-units. The latter possibility can be tested by size determinations of the normal and the 2-Me-DAB liver mitochondria and also by the determination in these mitochondria of the concentration of enzymes such as cytochrome c, which can be measured by methods other than activity determinations.

In the rats fed the carcinogen, 3′-Me-DAB, the changes that were observed in the number of nuclei and mitochondria or in the chemical and enzymatic properties of these livers were found to be explained either by the decreased amount of parenchymal cytoplasm in these livers or by the proliferation in the 3′-Me-DAB livers of bile duct epithelium or other cellular elements. Previous work on the mitochondrial fractions of normal and preneoplastic liver and of liver tumors (3, 7–12, 14, 17, 20) indicated that the mitochondria were involved in the carcinogenic process, since the amount of mitochondrial material and of mitochondrial enzymes was considerably less in the liver tumors and preneoplastic liver than in normal liver. More recent studies (22) have demonstrated, however, that mouse hepatoma and normal mouse liver cells contained about the same number of mitochondria, although the hepatoma cells were considerably larger than the normal liver cells and contained nuclei that were more polyploid than normal liver nuclei. Earlier studies on the biochemical properties of hepatoma and normal liver (3, 17, 20) indicated that the hepatoma mitochondria were grossly deficient in certain enzymatic functions present in normal liver mitochondria. This conclusion is not altered by the mitochondrial counts made on these tissues (22). The present results with the preneoplastic livers of the 3′-Me-DAB-fed rats indicate that the average number of mitochondria in the parenchymal cells of these livers was considerably less than in normal liver parenchymal cells, although the properties of the 3′-Me-DAB mitochondria were similar to those of the normal liver mitochondria. The picture that emerges from these studies is that, compared to the normal liver cell, the preneoplastic liver cell contains fewer mitochondria of approximately normal biochemical composition and at normal cytoplasmic concentration, while the hepatoma cell contains the same number of mitochondria of greatly altered biochemical properties and at reduced cytoplasmic concentration. It must be pointed out that these conclusions have been reached from studies on two species, the rat and the mouse, from extrapolations of measurements on large numbers of cells and without consideration of the fluctuations in mono- and binucleate cells. With these reservations in mind, it would appear that the data available at present support the concept that the mitochondria are involved in the carcinogenic process in liver, but it is also apparent, however, that the role of the mitochondria in this process is considerably more complex than the earlier data indicated.

SUMMARY

1. The livers of rats fed either a commercial chow diet, a semi-synthetic basal diet, as such, or supplemented with the noncarcinogenic azo dye, 2-Me-DAB, or the carcinogen, 3′-Me-DAB, were studied with respect to their nucleic acid content and their succinoxidase, DPN-cytochrome c reductase, octanoxidase, uricase, DNase, and RNase activities. The mitochondrial fractions of these livers were also isolated and analyzed for nucleic acids and enzymatic activity.

2. With the exception of DNase activity, the activity of the enzymes studied was lower in the livers of the rats fed the basal diet than in the livers of the rats on the chow diet. There was also less mitochondrial material in the basal livers than in the livers of the rats fed the chow diet.

3. The succinoxidase and DPN-cytochrome c reductase activity of the 2-Me-DAB livers was greater than that of the livers of the rats fed the basal diet, while the activity of the other enzymes studied was about the same in the 2-Me-DAB and basal livers. The amount of mitochondrial material was increased in the 2-Me-DAB livers, while the DNA and PNA concentrations in these livers was decreased slightly.

4. The DNase and RNase activities and the DNA concentration were considerably elevated in the livers of the rats fed 3′-Me-DAB, while the activity of the other enzymes studied was decreased.

5. The differences in the nucleic acid concentrations and enzymatic activities in these livers were correlated with and discussed in relation to the data on the number of mitochondria and nuclei and the quantitative morphological data presented in the previous paper.

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The Influence of 8-Azaguanine on the Mitotic Rate and Histological Appearance of Certain Normal and Neoplastic Tissues*

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It is known that the purine inhibitor, 8-azaguanine, is capable of inhibiting the growth of certain mouse tumors, two of which are mammary adenocarcinoma 755 and mammary adenocarcinoma E 0771, in C57BL mice (6, 7). We have suggested the hypothesis that this inhibition is brought about because 8-azaguanine is metabolized by the guanine enzyme systems of the tumor in place of guanine and is, therefore, incorporated into the nucleoproteins. Incorporation of 8-azaguanine into the nucleic acid of tumor and viscera of tumor-bearing mice (9) and into the ribonucleic acid of inhibited Tetrahymena (5) has been demonstrated. In order to test the hypothesis further, it seemed of importance to determine whether or not 8-azaguanine might have a selective action on the mitotic rate and histological appearance of tumors. It has been shown by Shapiro, Weiss, and Gellhorn (10) that "azaguanine has a specific effect on the mitotic activity of an undifferentiated squamous-cell carcinoma in rabbits (Brown-Pearce carcinoma)." They also found that the mitotic rate in the jejunum and testis remained unaffected by 8-azaguanine.

The present work extends these experiments to two mouse tumors which respond to 8-azaguanine (Carcinoma 755 and Carcinoma E 0771) and to four tumors which do not repond to the drug (Carcinoma 954, Carcinoma 1300, Sarcoma 91 and Sarcoma 180). Evidence will be presented that 8-azaguanine has a specific action on tumors 755 and E 0771; whereas this is not true of the other four tumors studied.

MATERIALS AND METHODS

By means of the trocar method, the following tumors were inoculated into susceptible hosts: tumors 755 and E 0771 into C57BL mice; tumor C 954 into leaden mice; tumor C 1300 into strain A hybrids; Sarcoma 91 into DBA/1 mice; and Sarcoma 180 into Swiss mice. In all cases, a random division into control and experimental groups was made only after substantial and measurable growth of the tumor could be observed. Half of the animals in each experiment were then treated with 8-azaguanine (32.5 or 65 mg/kg/day), while the other half served as controls. Each experiment was terminated when the first animal of the control group died; the remaining animals were then sacrificed. The first control with tumor 755 died 30 days after inoculation; with E 0771, 20 days; C 954, 29 days; C 1300, 31 days; S 91, 45 days; S 180, 27 days. Pieces of tumor, ileum, jejunum, and testis were fixed in Vandergrift's fluid and imbedded in paraffin, sectioned at 4 μ and stained with Harris hematoxylin and eosin. Seven and one-half hours prior to sacrifice, most of the mice were injected with colchicine at 3 mg/kg.

Mitotic counts were made under oil immersion. Both the total number of cells and the number of cells in mitosis were counted. In the case of tumor slides, a brief examination was made under the low power of the microscope in order to be sure of studying a healthy (non-necrotic) section of the tissue. Except for this, all fields examined were selected at random. Counts were made of four different fields in every case. The percentage figures in the tables are thus the average of these four counts.

RESULTS

Effects of 8-azaguanine on mitotic rate.—Table 1 indicates that both tumors 755 and E 0771 are significantly affected by 8-azaguanine, as judged by mitotic rate. In both of these tumors there are consistently more mitotic figures in the control tumors than in tumors treated with 8-azaguanine. In contrast to this, it is evident that 8-azaguanine has no inhibitory effect on the mitotic rate of the other four tumors studied (C 954, C 1300, S 91, and S 180). In one case, C 954, there was encountered a higher percentage of cells in mitosis in the treated than in the untreated tumor.

Evidence is presented in Table 2 that 8-azaguanine has no significant effect on the mitotic rate of ileum, jejunum, or testis. It may also be seen from Table 2 that the observation made by Guieysse-Pellissier (4) and Shapiro, Weiss, and Gellhorn (10), that colchicine does not seem to affect the mitotic rate of the testis, is confirmed. It is also evident that, in the other tissues, colchicine at the dosage level used does have the effect of

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preventing the completion of the mitotic process, thus causing an accumulation of metaphase figures.

In contrast to the results of Shapiro, Weiss, and Gellhorn (10) with the Brown-Pearce carcinoma of the rabbit, we were unable to find significant differences in the total number of tumor cells per oil immersion field when treated animals were compared to controls. Our results indicate that, in these six tumors, cell size remains approximately the same in treated and control tumors.

Effects of 8-azaguanine on histological appearance of tumors.—Histological study of Carcinoma 755 treated with 8-azaguanine revealed that, with increasing concentrations of the drug, there is an increase in the amount of fibrosis. This is indicated in Figures 1, 2, 3, and 4, in which a control tumor is contrasted with tumors which had been treated for 14 days. Staining reactions indicate that the material is collagen. This effect of the drug on tumor 755 is admittedly qualitative, but it is definite and reproducible. Attempts to find a similar difference between treated and untreated E 0771 tumors have not been successful; the same is true of tumors C 954, C 1300, S 91, and S 180. Ileum, jejunum, and testis of treated animals show no evidence of an increase in fibrosis when compared to untreated animals.

TABLE 1

THE EFFECT OF 8-AZAGUANINE ON THE MITOTIC RATE OF SIX MOUSE TUMORS

	Colchicine control				8-Azaguanine (65 mg/kg/day) and colchicine			
Tumor	No. of mice	Av. no. cells per oil immersion field	Av. no. mitoses per oil immersion field	Per cent mitoses per total no. of cells	No. of mice	Av. no. cells per oil immersion field	Av. no. mitoses per oil immersion field	Per cent mitoses per total no. of cells
755	6	233.8	7.72	3.3	10	213.4	1.67	0.8
E 0771	6	174.65	15.7	8.85	12	198.1	6.33	3.08
C 954	7	144.1	7.4	5.15	5	149.6	13.0	8.69
C 1300	11	175.1	20.3	11.62	6	156.8	16.5	10.52
S 91	13	153.0	8.61	5.63	19	152.68	9.89	6.48
S 180	9	137.0	6.66	4.86	11	139.18	6.72	4.83

TABLE 2

THE EFFECT OF 8-AZAGUANINE ON THE MITOTIC RATE OF NORMAL AND TUMOR 755 TISSUE IN MICE

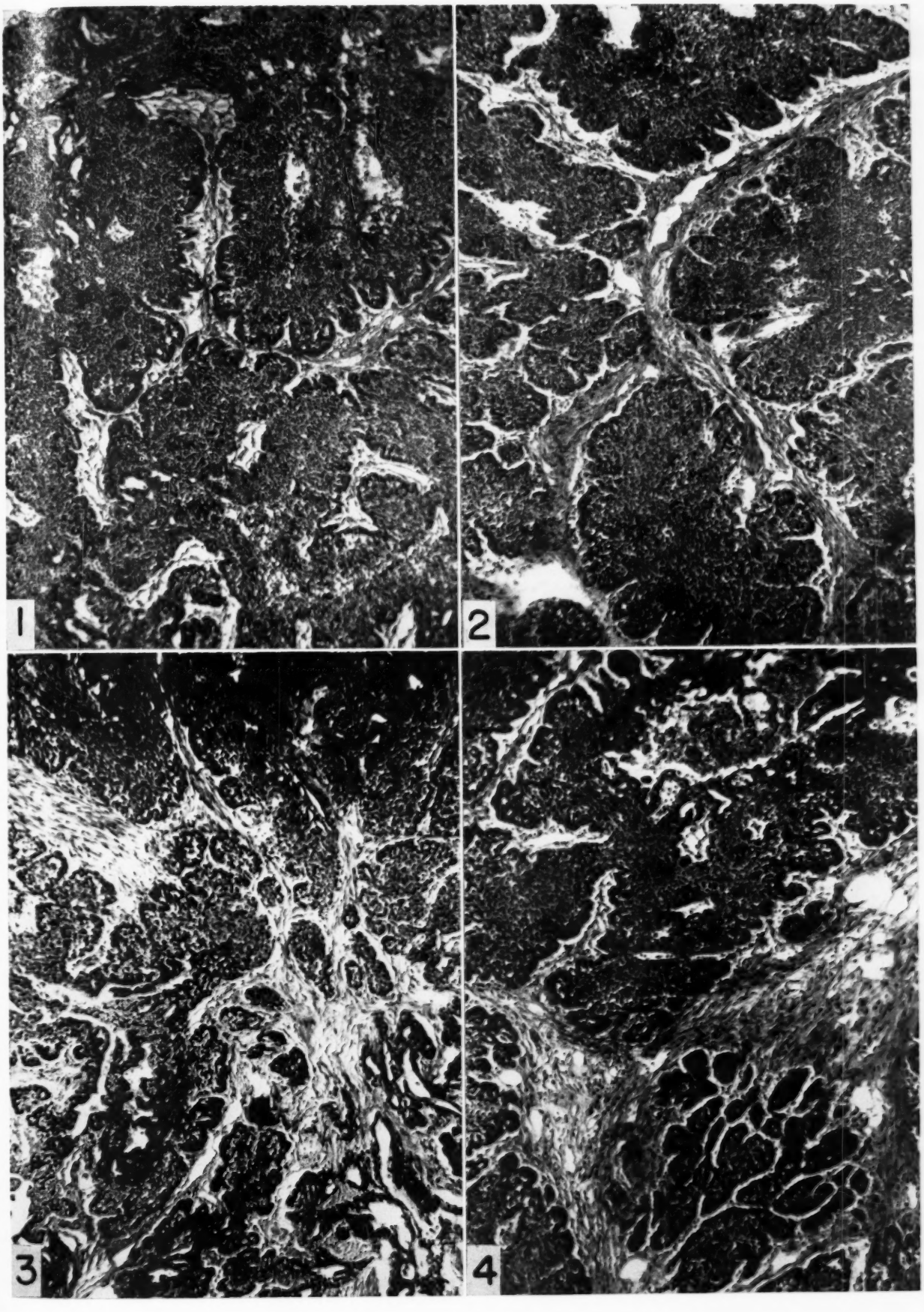
		Tumor				Ileum				Jejunum				Testis			
	Mouse no.	Cells per oil immersion field	Mitoses per oil immersion field	Per cent mitoses	Mean per cent mitoses	Cells per oil immersion field	Mitoses per oil immersion field	Per cent mitoses	Mean per cent mitoses	Cells per oil immersion field	Mitoses per oil immersion field	Per cent mitoses	Mean per cent mitoses	Cells per oil immersion field	Mitoses per oil immersion field	Per cent mitoses	Mean per cent mitoses
Control	782	183	2.02	1.1		161	3.75	2.32		178	3.0	1.68		223	60.2	26.9	
	786	213	2.02	0.95	1.0	236	7.0	2.96	2.6	201	5.5	2.73	2.2	241	56.8	23.6	25.2
Colchicine control	787	235	9.5	4.04		185	19.5	10.54		199	34.0	17.0		222	33.7	15.1	
	788	234	6.5	2.77		195	17.2	8.85		173	30.5	17.6		202	39.8	19.7	
	780	203	6.75	3.32		158	21.0	13.2		168	18.0	10.7		210	51.5	24.5	
	781	293	11.7	4.0		136	11.2	8.23		220	46.8	21.2		211	33.0	15.6	
	783	189	5.2	2.75		192	20.8	10.8		194	37.0	19.0		224	48.2	21.5	
	785	249	6.7	2.7	3.3	203	26.5	13.0	10.8	185	34.5	18.7	17.4	243	44.2	18.2	19.1
8-Azaguanine (32.5 mg /kg/ day) and colchicine	799	262	7.5	2.8		269	28.5	10.6		231	43.5	18.8		218	44.5	20.4	
	800	211	1.5	0.7		219	16.0	7.3		221	24.8	11.2		203	51.5	25.3	
	801	269	7.8	2.9		227	21.5	9.5		236	30.5	12.9		253	53.8	21.3	
	802	188	1.5	0.79		216	33.5	15.5		220	39.5	17.9		198	30.2	15.3	
	803	266	4.2	1.58		221	25.0	11.3		207	24.5	11.8		208	31.5	15.4	
	804	234	0.5	0.21		209	25.0	12.0		181	22.5	12.4		227	35.5	15.6	
	805	268	2.75	1.02		184	7.0	3.8		227	47.2	20.8		247	54.2	21.9	
	806	240	3.7	1.54	1.4	205	27.8	13.5	10.4	195	33.0	16.9	15.3	274	40.2	14.6	18.7
8-Azaguanine (65 mg/kg/day) and colchicine	789	274	1.25	0.456		243	45.2	18.6		189	32.0	16.9		211	39.0	18.5	
	790	212	1.5	0.707		181	23.8	13.1		169	17.0	10.0		249	33.8	13.6	
	791	205	0.75	0.366		214	39.0	18.2		177	23.8	13.4		261	46.2	17.7	
	792	204	3.25	1.59		207	32.2	15.6		127	22.8	17.9		233	44.8	19.2	
	793	191	1.0	0.52		180	27.0	15.0		168	24.8	14.7		205	36.5	17.8	
	794	184	1.0	0.54		172	24.5	14.2		155	21.0	13.5		221	28.0	12.7	
	795	214	3.0	1.4		191	23.8	12.4		178	29.0	16.3		196	37.2	18.9	
	796	195	1.75	0.897		191	30.0	15.7		181	38.0	20.9		188	35.2	18.7	
	797	208	1.0	0.48		171	22.2	13.0		179	28.0	15.6		197	33.5	17.0	
	798	247	2.2	0.89	0.8	174	26.5	15.2	15.1	234	30.5	13.0	15.2	234	54.8	23.4	17.7

FIG. 1.—Tumor 755. Control, no treatment. ×100.

FIG. 2.—Tumor 755. 8-azaguanine, 32.5 mg/kg/day for 14 days. ×100.

FIG. 3.—Tumor 755. 8-azaguanine, 65 mg/kg/day for 14 days. ×100.

FIG. 4.—Tumor 755. 8-azaguanine, 65 mg/kg/day for 14 days. ×100.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The effect of the presence of a tumor on the mitotic rate in other tissues.—Mitotic counts have been made on livers of mice bearing either tumor 755 or E 0771. Colchicine counts of mitoses in livers of tumor-bearing and nontumor-bearing mice (all untreated) reveal no differences in the two groups.

DISCUSSION

The experiments reported in this paper add to the evidence that 8-azaguanine has a selective action on mammary tumors 755 and E 0771 in mice. The conclusion drawn by Shapiro, Weiss, and Gellhorn (10) that "azaguanine has apparently both a direct and specific effect on the mitotic rate of neoplastic cells" is thus confirmed and extended to two of the six tumors studied in the present investigation. Mitotic inhibition is found only in the two whose growth can be inhibited by 8-azaguanine (755 and E 0771). Mitotic activity of the four tumors which are refractory to the drug (C 954, C 1300, S 91, and S 180) is not inhibited by it. Non-neoplastic tissues of all groups of animals remain unaffected by 8-azaguanine. These results indicate that the inhibition of growth brought about by 8-azaguanine in tumors 755 and E 0771 is the result of direct selective action on susceptible tumor tissue. Unlike the results reported by Shapiro, Weiss, and Gellhorn (10) for the Brown-Pearce carcinoma of the rabbit, we detected no significant difference in the total number of cells per oil immersion field between treated and control animals. This indicates that cell size in the six tumors studied remains unaffected by treatment with 8-azaguanine.

The only consistent histological effect other than that on mitosis was found to be an increase in the amount of fibrous connective tissue in tumor 755. It is possible that suppression of glandular growth resulted in a filling-in action of connective tissue.

It was reported by Annau, Manginelli, and Roth (1) that one rat tumor (a reticulo-endothelioma) and three mouse tumors (a fibrosarcoma and two mammary tumors) caused an increase in mitoses in the liver of animals bearing the tumor. These studies were apparently made without the aid of colchicine. In the present investigation, despite many attempts, we were unable to find a similar condition in the livers of mice with either 755 or E 0771. Colchicine counts of mitoses in livers of tumor-bearing and nontumor-bearing mice (all untreated) revealed no differences in the two groups. It seems, therefore, that these tumors do not produce substances capable of causing an increase in the mitotic rate of liver cells, at least in colchicine-treated animals. Studies on the tumors used by Annau, Manginelli, and Roth (1), utilizing the colchicine technic, would be valuable for purposes of comparison. That tumors can cause changes in non-neoplastic tissue has been shown by Cerecedo and associates (2, 3, 8), who demonstrated increased concentration of nucleic acids and derivatives in various organs of tumor-bearing animals.

SUMMARY

1. 8-Azaguanine caused a decrease in the mitotic rates of carcinomas 755 and E 0771 in mice.

2. The mitotic rates of mouse tumors C 954, C 1300, S 91, and S 180 were unaffected by the drug.

3. 8-Azaguanine did not change the mitotic rates of jejunum, ileum, or testis in mice bearing any of the six tumors studied.

4. Cell size remained the same in treated and control tumors whether or not the tumor was inhibited by 8-azaguanine.

5. One of the tumors studied, 755, developed an increased amount of fibrosis under the influence of 8-azaguanine.

6. With tumors 755 and E 0771, colchicine counts of mitoses in livers of tumor-bearing and nontumor-bearing mice (all untreated) revealed no differences between the two groups.

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The Metabolism of Methylated Aminoazo Dyes

III. The Demethylation of Various N-Methyl-C^{14}-aminoazo Dyes *in Vivo**

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The essentiality of at least one N-methyl group for the carcinogenic activity of the azo dyes related to 4-dimethylaminoazobenzene (DAB) has been well established (13, 15, 30, 31). Furthermore, the N-dimethyl and N-monomethyl dyes studied have essentially the same carcinogenic activities, while the primary aminoazo dyes are much less active (5, 16) and apparently owe their carcinogenicity to a small amount of N-methylation *in vivo* (16). On the basis of studies with DAB and 4-monomethylaminoazobenzene (MAB) (17), the equal activities of the mono- and dimethyl dyes are probably explained by a readily reversible demethylation of the N-dimethyl dyes. Demethylation of the N-monomethyl dyes also occurs readily, but in the cases examined it has been only slightly reversible (16, 17). The oxidative nature of the N-demethylation reaction has been shown by the isolation of equimolar amounts of formaldehyde and 3-methyl-4-aminoazobenzene following the incubation of 3-methyl-MAB with fortified rat liver homogenates (22). In accord with this observation is the rapid expiration of $C^{14}O_2$ following the administration of 4-dimethyl-C^{14}-aminoazobenzene or its 3′-methyl derivative to rats (2, 11). Studies on the 3′-methyl derivative further showed that a small but significant amount of the C^{14} was incorporated into the β-position of serine and the methyl groups of choline and thus also pointed to formaldehyde as an intermediate in the oxidation *in vivo* (11).

The introduction of ring substituents into DAB and MAB can either increase or decrease the carcinogenic activity of the dye (13, 15, 18, 20, 30). Since it seems likely that oxidized derivatives of the N-methyl dyes (such as the N-methylol compounds) may be more directly involved in the carcinogenic process than the N-methyl dyes themselves (9), the widely different activities of these ring-substituted derivatives could be related to alterations in the rates or pathways of metabolism of their N-methyl groups. In order to investigate this problem, several ring-methyl derivatives of DAB and MAB varying greatly in their carcinogenic activities were synthesized with C^{14} in the N-methyl groups and were administered to rats. The rate of expiration of $C^{14}O_2$ was used as an indicator of the over-all metabolism of the methyl groups, while the extents of incorporation of C^{14} into the β-carbon of serine and the N-methyl groups of choline were used as more specific indicators of formate or formaldehyde formation (1, 6, 24–28, 32). However, within the limitations of these methods, no differences in the metabolism of the methyl groups of strong and weak carcinogens or of N-dimethyl and N-monomethyl dyes could be detected.

METHODS

Synthesis of C^{14}-labeled dyes.[1]—Labeled DAB and MAB and their 4′- and 3-methyl derivatives were prepared by the reaction of methyl iodide-C^{14} with the appropriate primary aminoazo dyes to yield a mixture of the mono- and dimethyl dyes. For this purpose, 1 mM of methyl iodide-C^{14} (1 mc.) was partitioned by distillation under reduced pressure (3) among three pyrex tubes, each about 1 ml. in volume and containing about 4 moles of primary aminoazo dye per mole of methyl iodide added. After being sealed, the tubes were heated at 50° C. for 7.5 (in the case of 4-aminoazobenzene [AB]) or 27 hours. The dyes in each tube were then dissolved in ethanol containing slightly more ammonia than was required to neutralize the hydriodic acid formed in the reaction. This solution was evaporated to dryness, and the primary aminoazo dye and its mono- and dimethyl derivatives were separated by chromatography on a 1 × 24-inch column of aluminum oxide (12).[2] Each fraction was evaporated to dryness, the dye was weighed,

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[1] The authors are grateful to Dr. Charles Heidelberger for advice and assistance in the synthesis of the labeled dyes and in the counting of the radioactive samples.

[2] Merck aluminum oxide, "Suitable for Chromatographic Adsorption."

and the homogeneity of each dye was established by rechromatographing a small aliquot on aluminum oxide. The 3-methyl-MAB was rechromatographed to remove a reddish impurity, and the MAB was rechromatographed just before use, since it was slowly degraded to the primary aminoazo dye even in the solid state. The over-all yield of methylated dyes was 78 per cent on the basis of the methyl iodide-C^{14} used; the yield and specific activity of each dye are given in Table 1. The larger differences in the activities/mM of methyl group for the various dyes are apparently real, since replicate analyses in which the dyes were oxidized by either the Van Slyke-Folch reagent (3) or by combustion in "simple band" filled tubes in an oxygen stream at 550°–650° C. (23) had an average deviation of 5 per cent or less. The synthesis of N-methyl-labeled 3′-methyl-DAB with a specific activity of 4.7×10^6 counts/min/mg was described earlier (11).

The AB was a commercial sample (Eastman Kodak Co.), and the 4′-methyl-AB was synthesized from 4-methylnitrosobenzene and N-acetyl-*p*-phenylene diamine by the general method of Mills (21). 3-Methyl-AB was prepared by the general method of Mechel and Stauffer (7), except that the ω-methylsulfonate intermediate was isolated in solid form by a modification of the method of Eibner (4). For purification, the latter dye was converted to the hydrochloride, which was recrystallized twice from dilute hydrochloric acid; the free base was regenerated by reacting the hydrochloride with dilute ammonia. Each of these dyes was purified by chromatography on aluminum oxide prior to use.

In preliminary experiments with nonradioactive methyl iodide, the methylated derivatives were identified by chromatography alone and in mixture with the authentic compounds on aluminum oxide, by their characteristic colors in acid solution (12, 19), and by their melting points and melting points when mixed with the authentic compounds. Since the derivatives obtained from AB by this reaction had been characterized earlier as DAB and MAB (12), only the first two criteria were applied to these compounds. Ordinary melting point criteria could not be applied to the 3-methyl-DAB, since it is liquid at room temperature (15); however, in strong acid solution it had the yellow color characteristic of this derivative (19).

Care of the animals.—Young adult albino male rats[3] were used exclusively, except for two experiments in which either young adult or immature female rats were given DAB-C^{14}. At the time of administration of the radioactive dyes, the young adult rats weighed 190–250 gm. and the immature rats 69 and 73 gm. All the rats were fed ad libitum the semi-purified diet used routinely in this laboratory for the induction of liver tumors with the azo dyes. It consisted of: crude casein, 120 gm.; rice bran extract (Vitab[4]), 20 gm.; salt mixture, 40 gm.; corn oil, 50 gm.; glucose monohydrate, 770 gm.; halibut liver oil, 310 mg.; and sufficient riboflavin to make the total in the diet 1.5 mg/kg. The diet was fed either without the addition of dye (designated as the basal diet) or with the addition of 2.67 mM of nonradioactive dye/kg.

For each rat, enough radioactive dye to give the desired number of counts and sufficient of the corresponding unlabeled dye to bring the total dose to 10 μM of dye/100 gm of body weight were dissolved in 0.5 ml. of corn oil. The solutions were administered by stomach tube while the rats were under light ether anesthesia, and the animals were immediately transferred to metabolism cages similar to those described by Skipper *et al.* (29) for the collection of respiratory carbon dioxide, urine, and feces. Food and water were available ad libitum.

For the survey experiments on the general distribution of radioactivity, each of the dyes (approximately 10^6 counts/minute/rat) was administered to one rat prefed the basal diet and to another prefed the diet containing the unlabeled dye for 3 weeks. Respiratory CO_2 samples were collected at intervals over a 48-hour period. At the end of this time, the rats were killed with ether, and the urine, feces, and gastrointestinal tract contents were analyzed for radioactivity. For this purpose, the contents of the stomach and small intestine were separately flushed with water and ethanol and homogenized in the micro attachment of the Waring Blendor. The contents of the cecum and large intestine were combined with the feces and homogenized. Small aliquots of both of these suspensions were evaporated to dryness and oxidized with the Van Slyke-Folch reagent (3). The $C^{14}O_2$ samples from the oxidations and the respiratory $C^{14}O_2$ were counted as $BaC^{14}O_3$, and in both cases the observed counts were corrected for self-absorption and background and expressed as counts per minute on a standard counting instrument.

TABLE 1

THE YIELDS AND SPECIFIC ACTIVITIES OF THE C^{14}-LABELED N-METHYL DYES

Dye	Yield (mg.)	Per cent of the C^{14} incorporated	Specific activity: Counts/min/mg dye	Specific activity: Counts/min/mM of N-methyl group
MAB	44	21	5.1×10^6	10.8×10^8
DAB	16	14	9.0 "	10.1 "
4′-Methyl-MAB	25	11	4.2 "	9.3 "
4′-Methyl-DAB	22	17	7.0 "	8.4 "
3-Methyl-MAB	21	9	4.9 "	11.1 "
3-Methyl-DAB	7	6	*	

* Not determined.

In the experiments on the incorporation of C^{14} from the dyes into liver protein, serine, and choline, the rats were prefed the basal diet for 2 weeks. Each of the following dyes—DAB, 3′-methyl-DAB, 4′-methyl-DAB, and 3-methyl-MAB—containing 1.1 to 1.7×10^7 counts per minute was administered to each of two male rats, one of which was killed 5 and the other 10 hours later. 3-Methyl-MAB was used, since the low yield and

[3] Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

[4] National Oil Products Co., Harrison, N.J.

liquid state of 3-methyl-DAB largely precluded its use. The substitution seemed justifiable, since both dyes are extremely weak carcinogens and are apparently metabolized through a common pathway (19).[5] DAB containing approximately the same number of counts was also administered to each of two adult females and half of this amount to each of two immature female rats; these rats were killed 10 hours later. Choline, protein, and protein-bound serine were isolated from each liver (except that the livers from the immature rats were pooled and were not used for protein and serine isolations) after it was perfused *in situ* with ice-cold 0.9 per cent NaCl solution. The procedures for the isolation, degradation, and counting of these samples were described previously (11). Since the amounts of radioactivity administered varied somewhat from rat to rat, all the specific activities were converted to the "standard specific activities" which presumably would have been observed if exactly 10^7 c.p.m. had been given.

[5] J. A. Miller and E. C. Miller, unpublished data.

RESULTS

The general distribution of the radioactivity from DAB, MAB, and their ring-methyl derivatives following administration of the dyes to rats by stomach tube is shown in Tables 2 and 3. In

TABLE 2

DISTRIBUTION OF C^{14} IN THE RESPIRATORY CARBON DIOXIDE, URINE, AND FECES OF RATS FED CERTAIN N-METHYL-C^{14}-LABELED DYES

N-Methyl-C^{14}-labeled dye	Prefeeding of unlabeled dye	Per cent of total activity administered: Expired CO_2 5 hr.	Expired CO_2 10 hr.	Expired CO_2 48 hr.	Feces 48 hr.	Urine 48 hr.	Accounted for in 48 hr.
DAB	−	25	44	60	6	14	80
	+	25	51	66	4	16	86
MAB	−	25	38	48	5	18	71
	+	21	40	51	4	18	74
3′-Methyl-DAB	−	24	47	66	7	14	87
	+	23	42	56	5	21	82
4′-Methyl-DAB	−	21	35	46	14	17	77
	+	20	33	47	6	16	69
4′-Methyl-MAB	−	23	42	60	7	23	90
	+	22	37	49	8	35	92
3-Methyl-MAB	−	24	40	60	6	10	76
	+	20	38	59	9	14	82

TABLE 3

DISTRIBUTION OF RADIOACTIVITY IN RATS FED CERTAIN N-METHYL-C^{14}-LABELED DYES

	DAB					4′-methyl-DAB		3-methyl-MAB		3′-methyl-DAB	
	M	M	F	F	F*	M	M	M	M	M	M
Duration of experiments, hrs.	5	10	10	10	10	5	10	5	10	5	10
Per cent total activity:											
In respired CO^2, 5 hrs.	26	28	25	25	19	23	20	27	18	15	29
" , 10 "		46	47	48	36		40		41		54
In stomach and small intestine contents	13	8	5	3	8	20	8	12	7	27	5
In cecum and large intestine and feces	5	8	6	2	5	3	6	4	4	1	6
Standard specific activity† of:											
Liver protein	100	140	250	200		80	150	100	130	100	160
Liver serine (protein-bound)	1,000	1,400	3,400	2,300		750	2,000	530	980	350	1,600
Liver choline	4,600	5,000	7,200	6,000	9,400	2,800	5,300	4,900	4,000	3,100	5,700
Per cent activity:											
Of serine in β-carbon	98	104	100	100		99	95	93	94	93	99
Of choline in methyl groups	66	77	70	77	68	100	76	89	84	82	77

* Pooled samples from two immature rats; see "Methods."

† Standard specific activity (counts/min/mg) = $\frac{10^7 \times \text{observed specific activity}}{\text{activity administered (counts/min)}}$.

each case, C^{14} appeared rapidly in the expired CO_2; 30–50 per cent of the dose was expired in 10 hours and 50–70 per cent in 48 hours. During a 48-hour period, 4–9 per cent of the C^{14} was excreted in the feces and 10–30 per cent in the urine. These three routes of excretion accounted for 70–90 per cent of the C^{14} administered. Five hours after administration of the dyes, large and variable amounts (12–27 per cent of the dose) of radioactivity were found in the gastrointestinal tract; by 48 hours only small amounts could be detected.

As in our earlier study with 3′-methyl-DAB (11), the carbon atoms from the N-methyl groups of DAB, its 3′-methyl and 4′-methyl derivatives, and 3-methyl-MAB were incorporated into the protein, protein-bound serine, and choline of the liver to a small but significant extent (Table 3). The C^{14} from each of these dyes was utilized about equally well; this similarity is most readily seen in the samples taken 10 hours after administration of the dyes. In general, the specific activities of these samples were 50–100 per cent higher than those of the samples taken after only 5 hours and were more similar to each other. The greater variability observed at 5 hours was probably due, at least in part, to differences in the rates of absorption of the dyes by the individual rats. As in the previous study with 3′-methyl-DAB, all the C^{14} in the serine was in the β-position, and approximately 80 per cent (range 66 to 100) of the activity in the choline was in the N-methyl groups.

DISCUSSION

Since the carcinogenic activities of the ring-substituted derivatives of DAB differ greatly (13, 15, 18, 20), it seems reasonable to expect that detailed comparative analyses of the metabolism of a series of these dyes will aid in determining which metabolic reactions are essential to their carcinogenic activities. Such experiments have already pointed to the importance of chemical combinations between derivatives of the dyes and certain liver proteins in the carcinogenic process (10). These studies on the metabolism of the N-methyl groups were carried out, since a dye must have at least one N-methyl group to be a strong carcinogen and to yield high levels of protein-bound dye (8, 13, 15, 31). However, certain substituents, such as a methyl group in the 3 or 4′ positions, greatly diminish the activity of the N-methyl dyes and alter the pattern of bound dye formation with time, and it seemed possible that dyes with these substituents might have an altered rate or pathway of metabolism of the N-methyl groups. The present studies failed to indicate such a difference. Thus, the rate of expiration and over-all yield of $C^{14}O_2$ were approximately the same whether one of the strong carcinogens, DAB, MAB, or 3′-methyl-DAB, or one of the very weak carcinogens, 3-methyl-MAB, 4′-methyl-DAB, or 4′-methyl-MAB was administered. Likewise, the amount of C^{14} incorporated into the liver serine and choline was similar whether DAB, its 3′-methyl or 4′-methyl derivatives, or 3-methyl-MAB was given to the rat. Apparently, more detailed studies will be needed to detect any differences in the metabolism of the methyl groups of these dyes.

Presumably, most of the metabolism of the methyl groups of the dyes occurs in the liver. In experiments with fortified tissue homogenates, only preparations from liver demethylated the dyes at an appreciable rate, and this *in vitro* rate is far in excess of that observed *in vivo* (22). The liver homogenate system demethylates N-monomethyl dyes much more readily than N-dimethyl dyes, while no difference between these dyes was found with the *in vivo* system. Another difference was noted when normal rats were compared to those prefed a strong carcinogen such as 3′-methyl-DAB. Although liver homogenates from normal rats demethylate dye approximately 3 times as fast as homogenates from rats prefed the carcinogenic dye for 3 weeks,[6] rats from both groups expired $C^{14}O_2$ at approximately the same rate. Apparently, the demethylation system is not the rate-limiting factor *in vivo;* probably the rate of transport of the dye from the gastrointestinal tract to the liver is insufficient to allow the enzymes to work at capacity.

The specific incorporation of C^{14} from each of these dyes into the β-carbon of serine indicates that the methyl groups are oxidized at some stage via formaldehyde or formate. These one carbon compounds, but not carbon dioxide, have been shown by others (6, 24, 25, 27, 28) to be incorporated into serine exclusively in the β-position. This deduction is in accord with the finding of stoichiometric amounts of formaldehyde and primary aminoazo dye following the incubation of 3-methyl-MAB with fortified liver homogenates (22). From the *in vitro* experiments, a N-hydroxymethyl dye appears to be an intermediate in the oxidation; this dye is of particular interest, since theoretical considerations and model experiments suggest that it might be an intermediate in the formation of protein-bound dye in the liver (9).

In this and our previous study (11), we have consistently found small but significant amounts of C^{14} from the N-methyl groups of the dyes incor-

[6] R. R. Brown, J. A. Miller, and E. C. Miller, unpublished data.

porated into the methyl groups of the body choline. On the other hand, Boissonnas *et al.* (2) could not detect radioactivity in the choline isolated from a young female rat given N-methyl-labeled DAB. No explanation for this discrepancy has been found. We have used adult male rats in most of our studies, but the specific activities of choline samples isolated after the administration of radioactive DAB to adult or immature female rats were comparable to those obtained from male rats treated similarly. In our earlier study (11) the amount of incorporation was found to depend to some extent on the dietary conditions, but some incorporation was always obtained over a rather wide range of experimental conditions. While a small amount of transmethylation cannot be excluded, it is more likely that the radioactivity in the methyl groups of choline was derived from C^{14}-labeled formaldehyde or formate formed during the metabolism of the dye.

These data give no information on the stage of metabolism of the dye at which the N-methyl groups were oxidatively removed. Since other studies have shown that the level of AB in the blood is approximately the same whether DAB, MAB, or AB is fed to rats (17), most of the demethylation probably occurs prior to hydroxylation or reductive cleavage of the azo linkage of the dyes. However, since rats can also demethylate N,N-dimethyl-*p*-phenylene diamine, N-monomethyl-*p*-phenylene diamine, 4′-hydroxy-DAB, and 4′-hydroxy-MAB (14), any of these compounds which are formed during the metabolism of the dye probably, if demethylated, also yield C^{14}-labeled formaldehyde or formate.

SUMMARY

1. The synthesis of the following N-methyl-C^{14}-labeled dyes is described: 4-dimethylaminoazobenzene (DAB), 4-monomethylaminoazobenzene (MAB), and their 3-methyl and 4′-methyl derivatives.

2. Single doses of these labeled dyes (except 3-methyl-DAB) and N-methyl-C^{14}-labeled 3′-methyl-DAB were administered by stomach tube to rats prefed a semi-purified diet or the same diet containing the corresponding unlabeled dye for 3 weeks. In all cases the administered radioactivity appeared rapidly in the expired CO_2. By 48 hours 50–70 per cent of the C^{14} had been expired as $C^{14}O_2$, 10–30 per cent had been excreted in the urine, and 4–9 per cent had been excreted in the feces. The general metabolic pattern was the same, regardless of the dye used or whether the rats were fed a basal or dye-containing diet prior to the test dose.

3. In other experiments, protein, protein-bound serine, and choline were isolated from the livers of male rats given DAB, 3′-methyl-DAB, 4′-methyl-DAB, or 3-methyl-MAB; choline was also isolated from the livers of female rats given DAB. The specific activities of these liver components were essentially the same regardless of the dye fed or the sex of the rat. All the radioactivity in the serine was in the β-position, and, on the average, approximately 80 per cent of the activity in the choline was in the N-methyl groups. These *in vivo* data and other *in vitro* data indicate that the methyl groups of each of the dyes are oxidized to formaldehyde via an intermediate such as the N-hydroxymethyl dye.

4. Thus, within the limitations of these methods, no differences in the metabolism of the methyl groups of strong and weak carcinogens or of N-dimethyl and N-monomethyl dyes could be detected.

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Quantitative Relationship between Thyroid Function and Growth of Pituitary Tumors Secreting TSH*

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Destruction of the thyroid gland by I^{131} in mice is followed by the development of TSH (thyroid stimulating hormone)-secreting pituitary tumors (5, 11, 13). These tumors can at first be grafted only on mice similarly radiothyroidectomized and not on normal animals (5); thus, they are initially conditioned or dependent growths. The tumor cells gain autonomy in the course of passages, as indicated by their ability to grow in normal mice (6). In further subpassages in normal hosts, they grow even better in normal than in radiothyroidectomized hosts. These findings are best explained by successive modification in cells of the pituitary from normal to autonomous type.

The mechanism of induction of pituitary tumor by radiothyroidectomy is not known with certainty. It has been postulated that depression of the thyroid gland with depression in TH (thyroid hormone) secretion stimulates those cells of the hypophysis which control thyroidal function. The possibility that injury to the pituitary by radiations, incidental to radiothyroidectomy, is a contributing factor in the induction of pituitary tumors has, however, not been excluded thus far (10, 12). This problem calls for studies of minimizing irradiation of the pituitary incidental to I^{131} treatment or avoiding irradiation altogether by performing surgical thyroidectomies. The quantitative study of the interrelationship between thyroid function and pituitary growth reported in this paper aims to contribute to the understanding of the mechanism of induction of pituitary tumors and maintenance of their growth. It will answer the question whether dependency and autonomy are absolute or relative characteristics of tumors.

Dependent and autonomous tumors.—The induction and transplantation and some salient characteristics of the transplantable pituitary tumors have been described (5–7). Dependent tumor cells (Fig. 2) (those requiring absence of the thyroid for their growth) resemble normal chromophobe pituitary cells but are probably related to basophiles. They exhibit no features of anaplasia, even though they usually metastasize to regional lymph nodes. These characteristics have changed but little in the $1\frac{1}{2}$ years required for this work. The autonomous growths are more anaplastic and invasive. A conspicuous morphological difference between dependent and autonomous growths (Figs. 2, 3) relates to secondary changes in the tumors. Lack of secondary changes characterizes the dependent growth, while extensive necrosis, hemorrhage, and fibrosis are the rule with autonomous tumors. Anaplasia in the autonomous growths has increased markedly in the course of this study, and at its conclusion extensive lymphatic metastases in draining superficial and abdominal lymph nodes are now the rule. Nevertheless, the ability to secrete TSH, as indicated by enlargement of the thyroid (Fig. 4) and its microscopic appearance (Fig. 8), was invariably retained. (A normal thyroid is shown in Fig. 5.) A more detailed description of the morphological features and secondary changes of these tumors will follow.

MATERIALS AND METHODS

Pituitary tumors were grafted on mice, 6–15 weeks in age, that had been given a single dose of 25, 50, 75, and 250 μc. of I^{131} by subcutaneous injection when 5–8 weeks of age. Approximately 200 μc. destroys the thyroid, 50 and 75 μc. depress it, and 25 μc. produces little or no damage to this organ. After 3–6 months, the functional capacity of the thyroids of these animals was assayed by the administration of a few μc. of I^{131}.

C57BL mice of both sexes, either raised here or obtained from the Roscoe B. Jackson Memorial Laboratory, were used. Water and Purina chow, given ad libitum, were supplemented with carrots, lettuce, and hemp seeds. Tumor grafts were made by intramuscular injections of tumor particles in Tyrode solution.

Carrier-free I^{131} solutions, stabilized with

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$NaHSO_3$ and diluted with sterile 0.9 per cent NaCl, were injected in volumes of 0.2 ml. Following the injection of a near-tracer dose of I^{131} (~8 μc.), the precise amount of I^{131} injected and its retention at various intervals after injection were measured by placing the animal in the 100 per cent geometry gamma chamber (19). This instrument was also used to measure the radioactivity in various tissues at autopsy. *In vivo* the amount of radioiodine localized in the thyroid and other sites was estimated by a scintillation counter with a thallium-activated sodium iodide crystal. The mouse was placed at a distance of 3.5 inches from the crystal. Localization and collimation of radiations were achieved using lead shielding, 2 inches in thickness, with a cylindrical hole of 0.5 inch in diameter. An aluminum filter was interposed between the source and the crystal to exclude the beta rays of I^{131}. The error introduced by counting the activity in the tissues surrounding the thyroid was partially corrected by assuming that the volume of the thyroid region "seen" by the crystal was two-thirds that of the midbody region and that the activity not localized in the thyroid was equally distributed throughout the rest of the body. The scintillation counter was calibrated with I^{131} solutions standardized with the gamma chamber. All radioactivity measurements were adjusted for decay, with the time of injection being time zero.

Zenker-formol was the routine tissue fixative used. The pituitaries of tumor-bearing mice and pituitary tumors were stained with a combination of Martins and Mallory's trichrome in addition to hematoxylin and eosin stains. Tissues to be autoradiographed were fixed in Bouin's solution. The sections were placed on NTB plates 10 μ in thickness. Following exposure, development, and fixation, the sections were stained with Harris' hematoxylin and fast green.

Success of Transplantation in Relation to Graded Depression of the Thyroid with I^{131}

The relation between pretreatment with I^{131} and pituitary tumor growth was examined by grafting the tumors on mice that had received 25, 75, and 225 μc. of I^{131}, respectively. Four separate experiments were performed with our oldest dependent strain (3-D) and two with more recently isolated strains of tumor (19 and 124). The results of these experiments are shown in Charts 1–6.

In the first and fourth experiments (Charts 1 and 4), the pituitary tumors of strain 3-D grew rapidly in the mice pretreated with 225 μc. and very slowly in those given 75 μc.; no tumor growth was observed in mice given 25 μc. In later experiments, indicated in Charts 2 and 3, the 3-D tumors appeared also in the 25-μc. group but only after a long latency period. This was probably due to the acquisition of autonomy, as indicated by earlier experiments (6). The tendency to gain autonomy was marked with strains 3-D and 19. Growth of strain 19 tumors was proportional to the I^{131} dose (Chart 5). Susceptibility of mice to tumor strain 124 was markedly enhanced by pretreatment with 225 μc., slightly by 75 μc., and not at all by 25 μc. (Chart 6).

The depression of thyroid function was not uniform in mice that were given the same dose of I^{131}. Traces of thyroid were present in several mice in the 225-μc. groups and did not have an inhibitory effect on tumor growth. Great variations in thyroid depression in the 75 μc. groups were indicated by I^{131} uptake studies, histological examinations, and by enhancement of tumor growth. The function of these thyroid remnants was minimal, as will be shown later.

Functional Assay of the Thyroid Glands of Mice Given Graded Doses of I^{131}

5-Day study.—The depression or destruction of thyroid function by I^{131} was quantitated by giving near tracer doses of I^{131} and measuring the radioactivity which remained in the body and in the thyroid at 24-hour intervals. Some of the mice carried grafted pituitary tumors; others served as controls. The radioactivity localized in the thyroid region is shown in Charts 7 and 8. The values are expressed in terms of the percentage of the total dose injected and in terms of the total activity retained in the body, respectively. Chart 9 shows the total retention of I^{131} in the body.

The highest retention of I^{131} in the thyroid (Chart 7) occurred in normal mice bearing autonomous pituitary tumors that were large and rapidly growing (Group III). The next highest retention was found in normal mice bearing minute, usually microscopic tumor grafts of dependent strains over a long period of time (Group II). The thyroids of mice pretreated with 25 μc. (Group X), which had minute tumor grafts, retained more than those of normal mice without tumor grafts (Group I). It is remarkable how extensive stimulation of the thyroid is produced by secretion of surviving pituitary grafts of microscopic size. Very low retention in the thyroid was found in mice that had received 225 μc. (Groups IV and V, respectively), and most of this activity can be attributed to nonthyroidal tissue.

Since the total body retention of I^{131} differed considerably among the various groups, it was of interest to compare the retention in the thyroid in

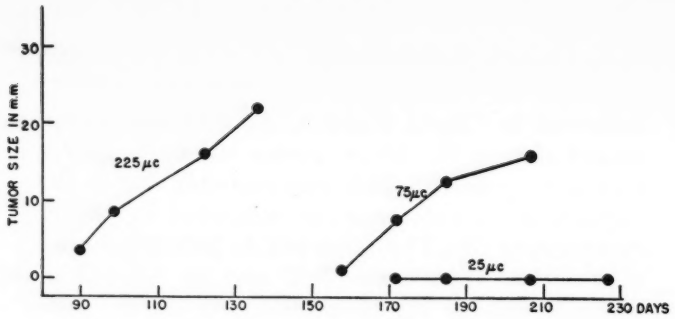


CHART 1.—The effect of pretreatment with 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 3-D). Each group was composed of four female mice.

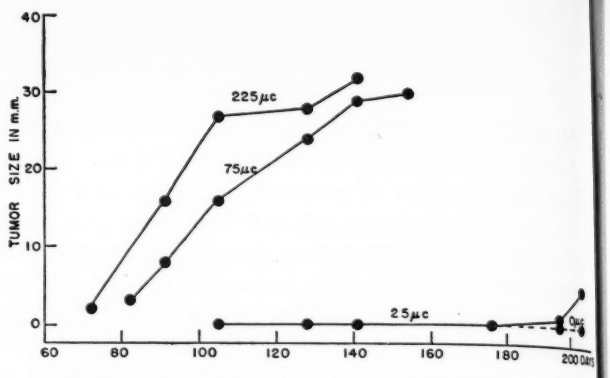


CHART 2.—The effect of pretreatment with 0, 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 3-D). Each group was composed of five male mice.

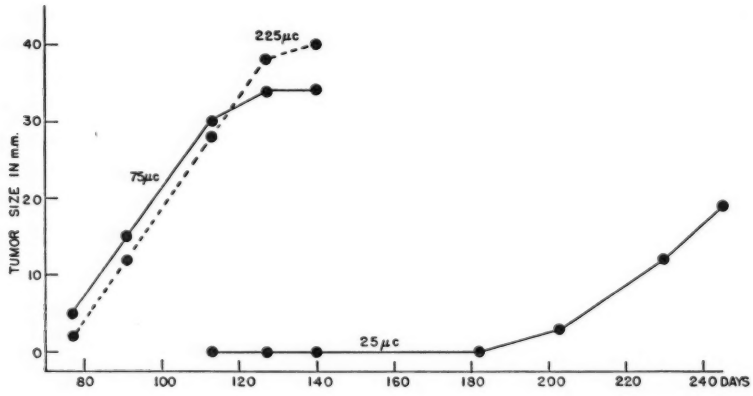


CHART 3.—The effect of pretreatment with 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 3-D). Each group was composed of five male mice.

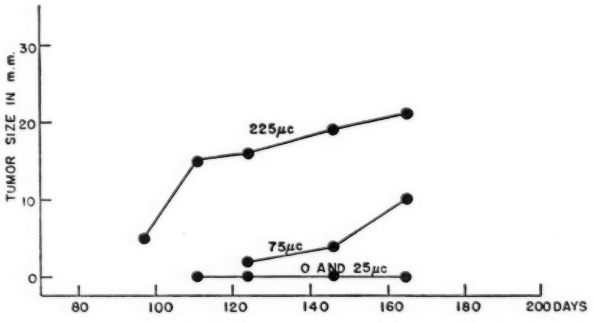


CHART 4.—The effect of pretreatment with 0, 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 3-D). Each group was composed of four female mice.

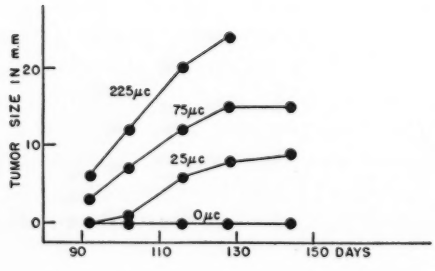


CHART 5.—The effect of pretreatment with 0, 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 19). Each group was composed of five female mice.

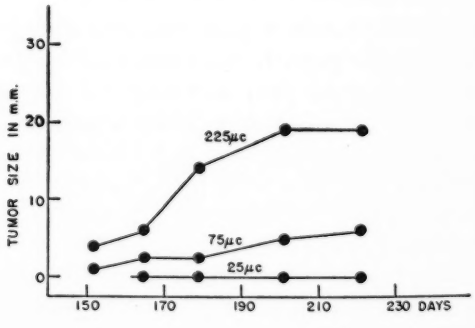


CHART 6.—The effect of pretreatment with 25, 75, and 225 μc. I^{131} on growth of grafted pituitary tumors (Strain 124). Each group was composed of four female mice.

Charts 1–6 show the relation of pituitary tumor growth to intensity of I^{131} pretreatment. The average tumor size in each group was plotted at various intervals from the time of grafting until the mice died with large tumors or were killed. In the event of death of animals within the group before the charted period, the tumor size at death was used thereafter.

relation to the total body retention (Chart 8). The curves representing the groups with pituitary tumor grafts fell in the same relative order as in Chart 7. In the normal and 25-μc. groups (I and X) the relative concentration of I^{131} in the thyroid increased until the fourth day following injection, after which it dropped. The curves for normal mice bearing tumors suggest a remarkable shift of I^{131} from the thyroid on the second day and to the thyroid on the third day. Further study would be required to establish fully the meaning of this shift.

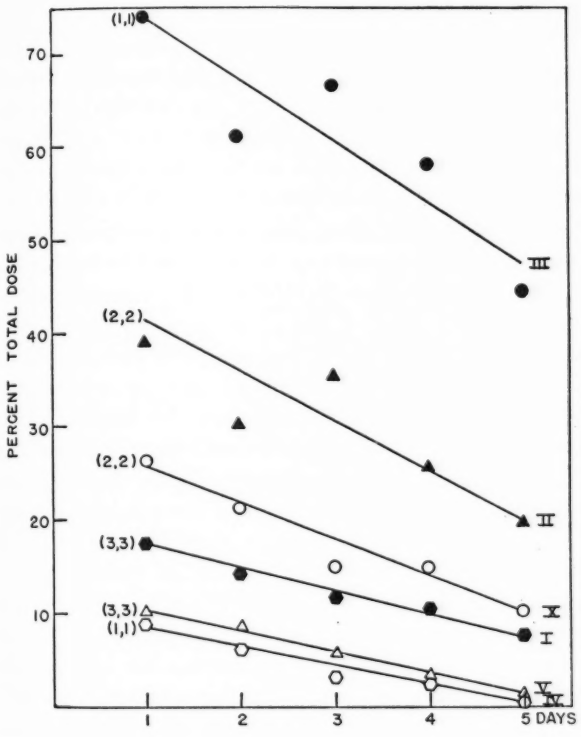


CHART 7.—Thyroid retention of a near tracer dose of I^{131}.

I. Normal nontumor-bearing mice.
II. Normal mice bearing minute grafts of dependent pituitary tumors.
III. Normal mice bearing large autonomous pituitary tumor grafts.
IV. Radiothyroidectomized mice not grafted with tumors.
V. Radiothyroidectomized mice bearing large dependent pituitary tumors.
X. Mice pretreated with 25 μc. of I^{131} and bearing minute dependent pituitary tumor grafts.

The first number in the parentheses indicates the number of mice in the group, the second the number of tests in the pooled data. About half the mice received two consecutive doses at an interval of 10 days.

The greatest 24-hour total body retention of I^{131} (Chart 9) occurred in radiothyroidectomized mice bearing large tumors, but this group (V) had also the most precipitous subsequent loss from the body. Radiothyroidectomy also enhanced the 24-hour retention of I^{131} (or reduced its elimination) in mice not given tumor grafts; this effect was, however, transient in these mice.

24-Hour study.—The ability of the thyroid hormone to inhibit the growth of pituitary tumors was further studied by microscopic examination of the thyroid glands following an assay of their functional capacity (Table 1). The mice were sacrificed 1 day following intraperitoneal injection of near tracer doses of I^{131}, since the results of the previous experiment (Charts 7–9) indicated that thyroid function can be evaluated by determining the 24-hour retention of I^{131}. Five experiments were performed with normal and I^{131}-pretreated mice with and without tumors. These were supplemented by experiments in which total-body retention of I^{131} was measured in the living animals by the gamma ray chamber and the retention in the thyroid by the scintillation counter. For comparison, similar tests were performed on mice that were given 25, 50, 100, and 200 μc. 7 months earlier but were not grafted with tumors (Groups IV, VI, VIII, IX).

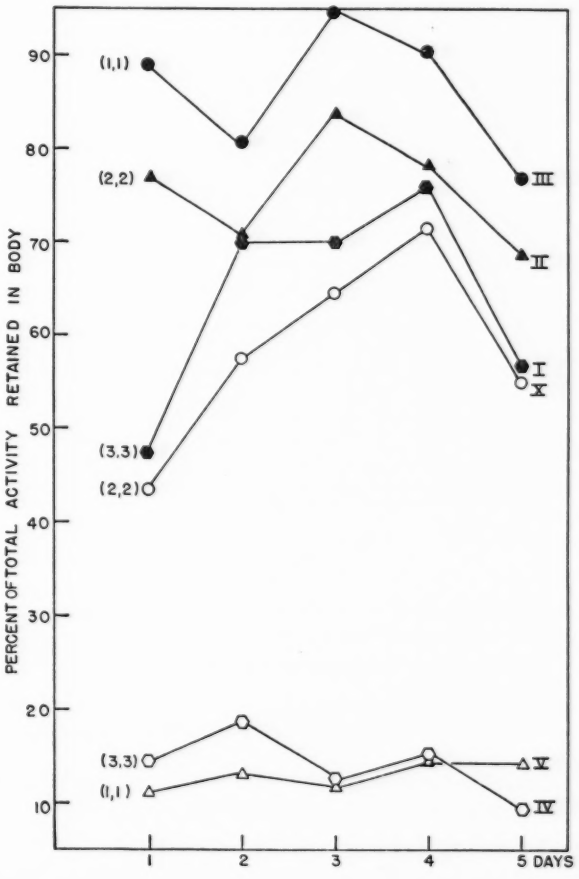


CHART 8.—Localization of a near tracer dose of I^{131} in the thyroid. See legend of Chart 7 for explanation.

All data are combined in Table 1. There was some variability in values, but the trend was the same in all experiments.

The mean total body retention in 24 hours in normal mice was 19.2 per cent of the tracer dose, and, of this, 70.5 per cent was in the thyroid gland (Group I). In contrast, the radiothyroidectomized mice (Group IV, Figs. 9, 10) retained 36.6 per cent of the injected dose in the body, but only a trace or none of the activity was in the thyroid region. The mean total-body retention in radiothyroidectomized mice bearing grafted tumors was 75.0 per cent of the dose (Group V), with only a trace or none at the site of the thyroid. The enhancing effect of grafted pituitary tumors on the uptake of radioiodine was thus confirmed.

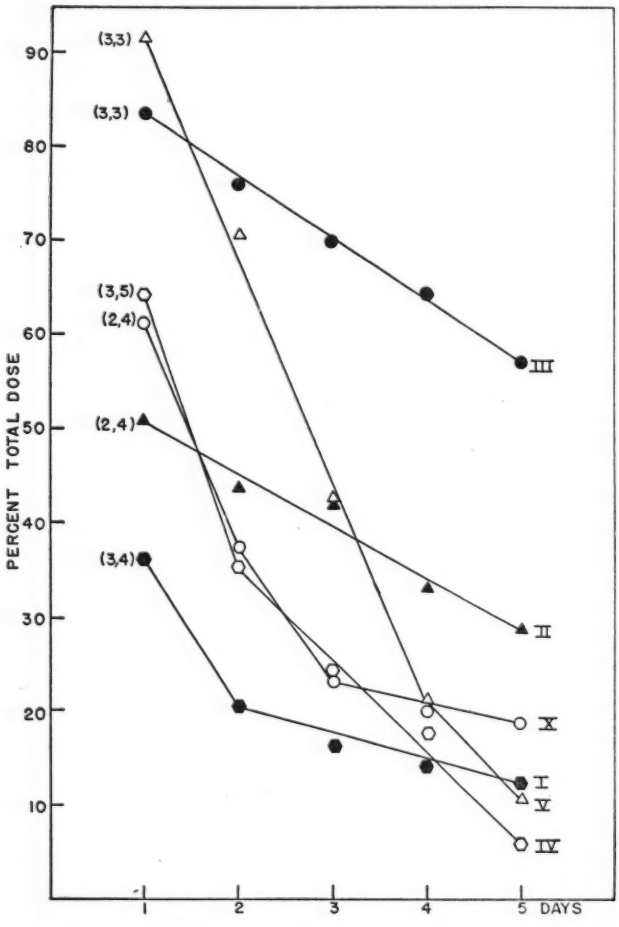


CHART 9.—Total-body retention of a near tracer dose of I^{131}. See legend of Chart 7 for explanation.

The total-body 24-hour retention of I^{131} was almost as much in normal mice bearing minute grafted dependent pituitary tumors (Group IIA) as in normal mice bearing large autonomous tumors (Group III). In Group IIA most of the radioiodine retained was in the thyroid gland (89.0 per cent), and most of this was presumably hormonal (TH) iodine. The tumor nodules in mice of this group were rarely larger than a normal pituitary gland at autopsy performed $4\frac{1}{2}$–9 months after grafting. The rapidly growing autonomous tumor grafts in the mice of Group III weighed at autopsy 2–5 gm., a thousand times that of a normal pituitary gland. Secretion of TSH by minute tumor grafts over a long period of time produces a striking stimulation with great enlargement of the thyroid gland.

The effects of pretreatment with 75 μc. of I^{131} on thyroid function of mice bearing tumor grafts were variable. There was, however, an inverse relationship between morphological appearance and function of the thyroid gland on the one hand and size of the tumor grafts on the other. This is clearly indicated by subdividing this group according to the quantity of I^{131} retention. In none of the four mice in which the 24-hour total-body I^{131} retention was above 50 per cent of the dose (Group VIIB) was the thyroid gland identified on gross examination, and in these mice the grafted tumors were large ($\backsim$25 mm. in average diameter). In Group VIIA the tumors were in general much smaller and the thyroids larger than in Group VIIB. While more I^{131} was retained in the body of the mice in Group VIIB (71.6 per cent) than in that of Group VIIA (43.7 per cent), only 4.7 per cent of that retained was in the thyroid in Group VIIB, as contrasted with 62.1 per cent in that of Group VIIA. These values indicate that 75 μc. injures the thyroid of different mice to a highly varying degree. The thyroids of all animals of this group had morphological changes indicative of stimulation by TSH and of varying grades of antecedent injury (Figs. 11–14). One was markedly fibrosed. A feature of the 75-μc. group was the co-existence of apparently resting and hyperstimulated follicles; similarly, the autoradiographs revealed a highly variable uptake of I^{131} in different follicles.

The behavior of the mice in the 25-μc. group bearing minute tumors was less variable than that of the 75-μc. group. The two mice with the lowest I^{131} uptake by the thyroid (Group XB, Fig. 16) had larger tumor grafts than those of XA, so that it is probable that 25 μc. of radioiodine injured the thyroid gland of these mice. If the remaining thirteen of the fifteen mice receiving 25 μc. (Group XA) are compared to the 0-μc. group bearing minute tumors (IIA), it is noted that the I^{131} retention was about the same in both (60.8 and 61.9 per cent, respectively), and most of the I^{131} retained was in the thyroid (86.1 and 89.0 per cent). Thus, the conclusion is warranted that in these thirteen mice 25 μc. did not noticeably alter the thyroid.

Whether or not 25 μc. had injured the thyroid could also be evaluated by comparing normal mice (Group I) to those given 25 μc. but not grafted with tumor (Group IX, Fig. 15). The I^{131} retention in the body and the thyroid was only slightly greater in Group IX. This can be explained by assuming that this dose slightly depresses the thyroid function of some mice.

The possibility that the enhanced total body retention of I^{131} in tumor-bearing radiothyroidectomized animals was due to specific localization of I^{131} in the tumors was examined by determining the I^{131} uptake in tumors following the administration of near tracer doses. The data show that grafted pituitary tumors do not have a specific affinity for iodine (Table 2). A comparison of these values with those of Rugh (16, 17) on the uptake of I^{131} by different organs indicates that the uptake of I^{131} by tumors is within the range of that of organs other than the thyroid.

The amount of I^{131} in the circulating blood 24 hours after injection was also small. In four radiothyroidectomized mice it ranged from 4.6 to 7.3 per cent of the amount injected; in normal mice it was less than 1 per cent. A large part of the total activity retained was found in the intestine, but the site of most I^{131} retained in radiothyroidectomized animals was in the eviscerated carcass.

Morphological Observations on Thyroid Function of Mice Receiving Graded Doses of I^{131}

The effects of radioiodine on the thyroid can be fully understood only by a consideration of a dynamic state covering the life span of the animal from the time of treatment until natural death. Depression of the thyroid can be assumed to be followed by stimulation of the pituitary with a secondary stimulation of the thyroid. The functional state of the injured thyroid can be expected to vary with time. A study is in progress to assess the long-term effects of graded doses of radioiodine on mice; function and morphology are being correlated with tumor development.

TABLE 1

TOTAL BODY AND THYROID RETENTION OF NEAR TRACER DOSES OF I^{131} IN NORMAL AND I^{131} PRETREATED MICE BEARING TUMORS AND IN CONTROLS

Group	No. mice in group	Original dose of I^{131} (μc.)	Type of tumor grafted	Tumor size*	Thyroid size	Per cent of I^{131} retained in 24 hours: In body, Per cent injected, Av.	In body, Per cent injected, Range, Low	In body, Per cent injected, Range, High	In thyroid, Per cent injected, Av.	In thyroid, Per cent injected, Range, Low	In thyroid, Per cent injected, Range, High	In thyroid, Per cent in body, Av.	In thyroid, Per cent in body, Range, Low	In thyroid, Per cent in body, Range, High
I	18	0			Normal	19.2	10.2	39.4	14.5	4.6	41.1	70.5	42.9	100.0
IIA	10	0	Dependent	Minute	Marked increase	61.9	30.3	86.8	56.2	24.6	82.2	89.0	75.9	97.3
IIB	3	0	Dependent	Small	Marked increase	82.2	81.8	82.7	82.2	74.2	86.6	96.8	90.4	100.0
III	6	0	Autonomous	Large	Marked increase	75.5	63.8	89.9	75.5	55.9	97.7	87.0	67.3	94.3
IV	10	200–300			Absent or rudimentary	36.6	13.6	73.4	<1.0	0.0	3.1	<1.0	0.0	2.4
V	8	200–300	Dependent	Large	Absent or rudimentary	75.0	57.1	91.7	<0.1	0.0	0.1	<1.0	0.0	3.9
VI	9	100			Rudimentary or small	10.9	4.1	19.2	<1.0	0.0	2.2	<2.0	0.0	7.9
VIIA	4	75	Dependent	Minute	Small	43.7	29.2	49.9	28.5	10.0	39.5	62.1	34.2	81.9
VIIB	4	75	Dependent	Large	Minute	71.6	50.7	92.1	2.8	1.4	5.5	4.7	1.7	10.8
VIII	8	50			Small	14.5	4.9	22.1	10.1	0.7	18.3	63.0	14.6	87.3
IX	8	25			Normal	28.6	15.5	48.4	23.1	12.5	43.2	79.7	53.9	100.0
XA	13	25	Dependent	Minute	Slight to moderate increase	60.8	28.0	71.7	30.5	6.6	61.6	86.1	23.5	97.2
XB	2	25	Dependent	Small		38.7	30.2	47.1	23.6	14.5	32.7	58.8	48.1	69.5

*Minute = measuring 1–3 mm. in average diameter; small = approximately 1 cm.; large = 2–3 cm. The subdivision of Groups II and X was made on basis of tumor size: Mice of Group A had minute tumors not detectable by palpation and those of Group B had small palpable tumors. The 75-μc. group was subdivided according to the I^{131} retention: Group VIIA is composed of animals retaining less than 50 per cent and Group VIIB more than 50 per cent of the injected dose.

TABLE 2

ASSAY OF IODINE RETENTION OF TSH-SECRETING PITUITARY TUMORS WITH NEAR-TRACER DOSES OF I^{131}

Original dose of I^{131} (μc.)	Type of tumor grafted	Weight of tumor (gm.)	Per cent of tracer dose retained: Total body	Tumor	Thyroid
0	Autonomous	1.9	69.8	0.3	63.7
0	Dependent	0.3	82.0	0.1	74.2
0	Autonomous	~4	84.5	2.2	76.7
75	Dependent	2.5	85.5	2.4	1.5
225	"	3.6	57.1	3.0	0.0
225	"	1.0	70.5	1.4	0.0
400	"	~4	51.8	5.3	0.0

To provide controls for the tumor-bearing mice pretreated with graded doses of I^{131}, similar mice without tumors, available 7 months after the administration of graded doses of I^{131}, have been included in the present study (Table 1). The thyroids of the controls (Group IX) that were given 25 μc. exhibited subtle changes which could be detected only by a close comparison with normal thyroids (Figs. 6, 7). There was a slight increase in

size of the thyroid cells, notably at the periphery of the gland, with some resolution of the colloid (Fig. 15). The uptake of I^{131} by thyroids of mice pretreated with 25 μc. averaged 23.1 per cent of the injected dose, as contrasted with 14.5 per cent in normals. In animals that had received 25 μc. and bore pituitary tumor grafts, injury was indicated by slight fibrosis and occasional slight atrophy, and stimulation by swelling of the epithelial cells. The latter contained many intracytoplasmic vacuoles and colloid droplets and exhibited some features of anaplasia. With tumors of long duration, adenoma formations were common (Figs. 17, 18).

Fifty and 75 μc. of I^{131} produced conspicuous changes consisting of slight to moderate fibrosis, occasional atrophy, moderate to marked variability in the size and shape of the cells and acini and in quantity and staining quality of the colloid (Figs. 12, 13). The lining cells of some acini were flat; others were greatly swollen, and frequently the same follicle was lined with cells of greatly varying sizes. There were marked differences in size and chromatophilia of the nuclei. Some were small, others "giants," some normochromic or hyperchromic with respect to affinity for hematoxylin. The cytoplasm of many cells was filled with colloid droplets varying greatly in size and number. Most cells were intensely vacuolated.

One hundred μc. of I^{131} produced a marked reduction in the number of cells of the thyroid so that the organ could not be identified at a magnification of 5 × at autopsy. Microscopic examination disclosed a more intense change in the residual thyroid than in mice of the 50- and 75-μc. groups. Anaplasia as concerns nuclear size, shape, and chromatophilia was particularly marked. Note that 50–100 μc. markedly depressed the thyroidal uptake of I^{131}, but, in the presence of tumor, I^{131} uptake was fairly high (75-μc. group). The presence of tumor markedly increased the total body retention of I^{131} (estimated 12–50 per cent of the injected dose), and this enabled a greater uptake of I^{131} by the small residual thyroid tissue that had been stimulated by the TSH secreted by the pituitary tumors.

Two hundred and fifty to 400 μc. of I^{131} destroyed the thyroid almost completely. Its site was identified by a marked stenosing "radiation arteritis," often with perivascular infiltrations by lymphoid cells and hemosiderin-filled macrophages in an area of fibrosis adjacent to the parathyroid (Figs. 9, 10). Frequently, with a 200-μc. dose, a few isolated acini remained, exhibiting the retrogressive changes already described, anaplasia being particularly marked. The presence of these rudimentary thyroid cells can be readily detected *in vivo* by the scintillation counter following administration of a tracer dose of I^{131}. Thus, they function, but are unable to undergo compensatory hyperplasia when constantly stimulated by the tremendous quantities of TSH discharged by the grafted tumor. In mice receiving 300 μc. or more, such rudimentary thyroid tissue was but rarely identified.

Thus, the results of the morphological and iodine uptake studies are in agreement. The threshold dose of I^{131} for depressing the thyroid of mice appears to be in the neighborhood of 25 μc. This dose might cause a slight depression or even an apparent stimulation in some animals. Correspondingly, this is also the threshold dose for rendering some animals susceptible to tumor growth; in others the grafts are merely held *in situ* as they are in normal hosts.

DISCUSSION

While it is felt that the prime aim of this study has been attained, several deficiencies in technic were noted *post hoc;* pointing these out will be helpful in future research on related problems. Not anticipating the importance of knowing precisely the size of the thyroid, this organ was not weighed. Careful inspection under the binocular microscope might identify thyroid remnants. Weighing and serially sectioning this organ as well as weighing the tumors and the pituitary post mortem will add to the accuracy of such work. Autoradiography should be performed on tissues dehydrated in the frozen state in order to minimize loss of I^{131}.

Correlation of function with tumor growth.—One objective of this study is to correlate thyroid function with growth of TSH-secreting pituitary tumors. The 24-hour uptake of I^{131} by the thyroid is a good index of the functional capacity of the thyroid in mice. Hyperthyroidism (TSH excess) increases the thyroid uptake of I^{131} in mice as it does in man (4); hypothyroidism reduces it, and zero values are obtained in athyroidism. There is an excellent correlation between depression of thyroid function and growth of grafted dependent pituitary tumors. Individual differences are evident in all groups, and are greatest in the 75-μc. group (Figs. 11–14). These variations can be attributed in part to technical inaccuracies in amounts injected, and in part to individual variations in response. Whatever its cause, this variability was an aid in correlating thyroid function with tumor growth; e.g., all mice of the 75-μc. group in which the uptake was more than 50 per cent had gross tumors, and those with an uptake of I^{131} of less than 50 per cent had no tumors.

A correlation between morphology and functional capacity of the thyroid was usually evident; e.g., in the 75-μc. group the mouse with the highest I^{131} uptake had, on microscopic examination, the

largest and most stimulated thyroid of the group. There are ample data on the quantitative relationship between TSH and thyroid stimulation (1, 9, 15); the present study supplements these by correlating thyroid function with stimulation of growth of the pituitary cells secreting TSH. A complete review of the thyroid-pituitary relationship is beyond the scope of this paper; there are many excellent recent articles on this subject (cf. 1, 2, 4, 9, 15).

The present studies and those of Rugh (16) indicate that complete destruction of the thyroid is not necessary for induction of pituitary tumors. There is no clear-cut evidence of new formation of follicles after partial radiothyroidectomy; however, there is ample evidence that some cells surviving partial radiothyroidectomy are hyperfunctioning.

The failure or tardiness of the thyroid gland partially destroyed by small doses of I^{131} to undergo fully compensating hyperplasia deserves emphasis and further study. This may be a radiation effect; in man, partial removal by surgery is known to be followed by regeneration. One noteworthy difference between surgical and radiothyroidectomy is the stenosing arteritis and fibrosis caused by irradiation, which alone may explain failure of regeneration. Comparative studies on regeneration after surgical and radiothyroidectomy are desirable.

No close correlation could be made between the results of tests on the uptake of I^{131} and the autoradiographs of the same thyroids. This partial failure can be attributed to the loss of I^{131} in the course of processing, as pointed out by Holt and Warren (14), and might be avoided by the use of the freeze-dry technic (14). Nevertheless, the loss in wet processing was not great, and the autoradiographs furnished some confirmatory data on the degree of I^{131} retention and served to establish the uniformity of distribution of I^{131} or the lack of it. It is well known that morphologic appearances may be misleading. Follicles in thyroid may contain colloid yet be functionally inactive; tumors may appear well differentiated yet highly invasive (2). Autoradiographs have indicated the heterogeneity of acini in thyroids injured by I^{131}. Adenomas caused by sustained stimulation of the thyroid by TSH-secreting tumors failed to take up demonstrable amounts of I^{131} in the presence of normal-appearing acini which accumulated I^{131}. In mice given large doses of I^{131} thyroid remnants composed of vacuolated atypical cells could be definitely identified as such by their effect on the photographic emulsion.

If the growth stimulus of pituitary tumors is lack of TH, blocking TH synthesis with thiouracil might also be expected to render normal mice susceptible to pituitary growth; administration of thyroid hormone ought to abolish susceptibility caused by radiothyroidectomy. Studies to be reported later indicate both to be true. Thiouracil has proved, however, to be much inferior to complete radiothyroidectomy, and its effect is equivalent to partial radiothyroidectomy as caused by approximately 50 μc. of I^{131}. Induction of pituitary tumors by administration of thiouracil in mice has also been reported (18) but not confirmed. That administration of TH will prevent the induction of primary pituitary tumors has already been shown by Gorbman (13) and Goldberg and Chaikoff (10); similarly, tumors grafted in radiothyroidectomized mice can be restrained by TH. Thus, there is some parallelism between conditions influencing induction of pituitary tumors and their transplantability.

Hormonal secretion of the pituitary tumors and its relation to tumorigenesis.—Knowledge of the type and quantity of secretions is essential for the understanding of the problems under consideration. Several investigators have independently established by bioassays the secretion of TSH by these tumors (Dent,[1] D'Angelo,[2] Halmi[3]), and their findings have indicated the discharge of large quantities of TSH by the tumors. Most valuable are the assays of Anderson[4] in hypophysectomized mice which likewise indicate secretion of TSH by the tumors and, in addition, absence of changes indicative of secretion of other hormones in significant amounts. Normal mice bearing autonomous pituitary tumor grafts have tremendously enlarged and stimulated thyroids. Changes in the thyroid of mice that received 50 to 100 μc. of radioiodine without tumor grafts were similar to those seen in normal mice bearing autonomous pituitary tumors. It can be assumed that partial radiothyroidectomy causes stimulation of TSH secretion by the pituitary, with secondary hyperfunction of the thyroid cells whose proliferative capacity is impaired by the irradiation. In mice that had been given 100 μc. and larger doses 7 months earlier, microscopic examination of the hypophyses showed nodular hyperplasia of chromophobe cells, which is known to precede tumor development. Similar alterations were absent in the hypophyses of mice given 25 and 50 μc.

The relationship between the development and growth of pituitary tumors and thyroid function

[1] Personal communication with Dr. James N. Dent.

[2] Personal communication with Dr. S. A. D'Angelo.

[3] Personal communication with Dr. N. S. Halmi.

[4] Personal communication with Dr. Evelyn Anderson.

applies only to those pituitary tumor cells which secrete TSH. It is possible that a similar relationship exists between pituitary cells secreting other types of hormones and the function of the stimulated organ. The problem of tumor induction is how to disturb the specific normal balance between the pituitary and its target organ. Pituitary tumors have been described in gonadectomized mice (3), but the functional capacity of these tumors and their specific relationship to the gonads have not been adequately studied. Compensation by the adrenal gland for lack of gonadal hormone doubtless interferes with the development of pituitary tumors following gonadectomy. Adrenalectomized mice have not to our knowledge been maintained long enough for the development of tumors in the pituitary gland. Pituitary tumors have also been induced in C57BL mice by long continued administration of estrogens (cf. 8). This and other related pioneer research are being surveyed and discussed in a review on conditioned and autonomous neoplasms.[5] It is noteworthy that all work on the induction of pituitary tumors by radiothyroidectomy reported thus far was done with C57BL mice.

Genetically controlled strain and species differences in susceptibility may be additional factors influencing the development of pituitary tumors following radiothyroidectomy. The possible role of irradiation as an accessory factor has been suggested by Gorbman (12). By keeping mice on an iodine-free diet he could reduce to 30 μc. the quantity of radioiodine needed to destroy the thyroid. However, reducing iodine in the diet will enhance the uptake of radioiodine in the thyroid, and consequently the dose of irradiation received by the thyroid will equal that of animals kept on a normal diet and given much larger doses of radioiodine. The statement that thyroidectomy is not followed by the development of pituitary tumors (16) has to our knowledge never been documented; we found no report in the literature on chronic effects of thyroidectomy in mice. Thus, it is uncertain whether or not irradiation of the pituitary coincidental to radiothyroidectomy is essential to induction of pituitary tumors.

Enhancement of body retention of I^{131} after radiothyroidectomy.—An explanation is wanted for the enhanced total body retention of I^{131} in radiothyroidectomized mice. This procedure nearly doubled I^{131} retention, and the tumor graft further doubled this under our experimental conditions.

It is known that TSH decreases the excretion rate of I^{131} (9), but in normal hosts this can be attributed to increased synthesis of TH. Studies on the uptake of I^{131} indicate that the tumors have no special affinity for I^{131}. The exact localization of I^{131} in radiothyroidectomized mice as well as the chemistry of compounds carrying it remain to be studied. It is probable that radiothyroidectomized mice have an increased amount of circulating TSH. Pituitary tumor grafts raise the TSH blood level, and it is perhaps this hormone itself which modifies the iodine retention capacity of the body. These assumptions call for quantitative studies of levels of TSH in relation to iodine retention in the body and partition of substances containing io-

[5] To be published.

All sections were stained with hematoxylin and eosin. The magnifications are approximate.

FIG. 1.—Gross appearance of a mouse with large dependent pituitary growth (Strain 3). Arrows point to large seminal vesicle, cystic dilatation of the biliary tract, and metastases in iliac lymph node.

FIG. 2.—Dependent pituitary tumor of Strain 3. Note uniformity of cells and absence of anaplasia. ×240.

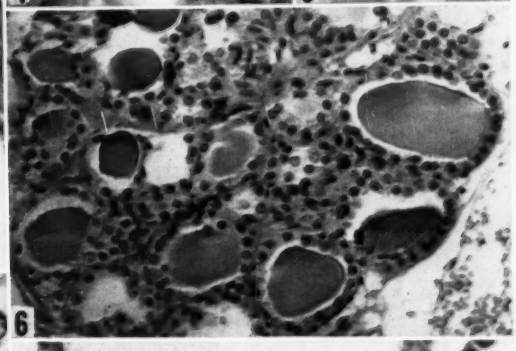
FIG. 3.—Autonomous pituitary tumor of Strain 3. Note the variations in size, shape, and staining intensity of the cells, and regressive changes. ×100.

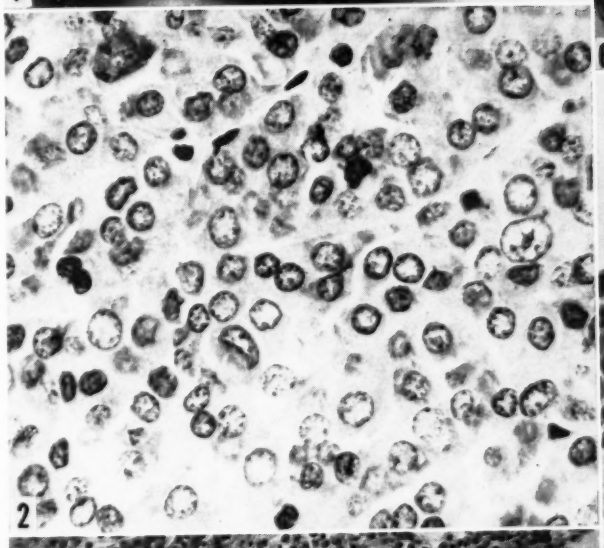
FIG. 4.—Gross appearance of an enormously enlarged thyroid gland of a noniodinated mouse bearing a small pituitary tumor 10 months after graft (Strain 3; late autonomy). ×4.5.

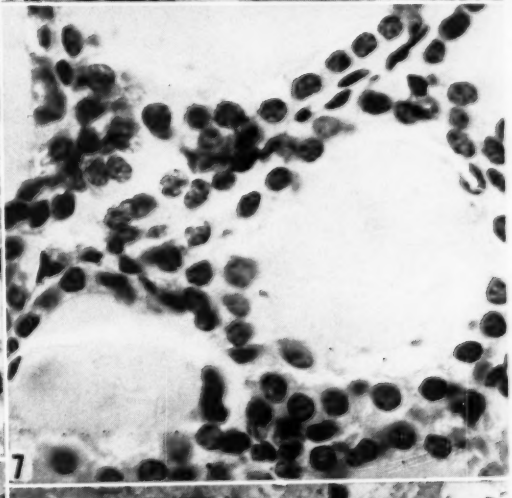
FIG. 5.—Normal thyroid shown for comparison with Figure 4; arrow points to the left lobe of the thyroid. ×4.5.

FIGS. 6 and 7.—Microscopic appearance of the normal thyroid of the strain of mice under study. ×100 and ×240, respectively.

FIG. 8.—Hyperstimulated thyroid of a normal mouse carrying a large autonomous pituitary tumor graft (Strain 3). Note the swelling of cells, resolution of colloid, and intracytoplasmic globules of colloid, the latter indicated by arrows. ×970.







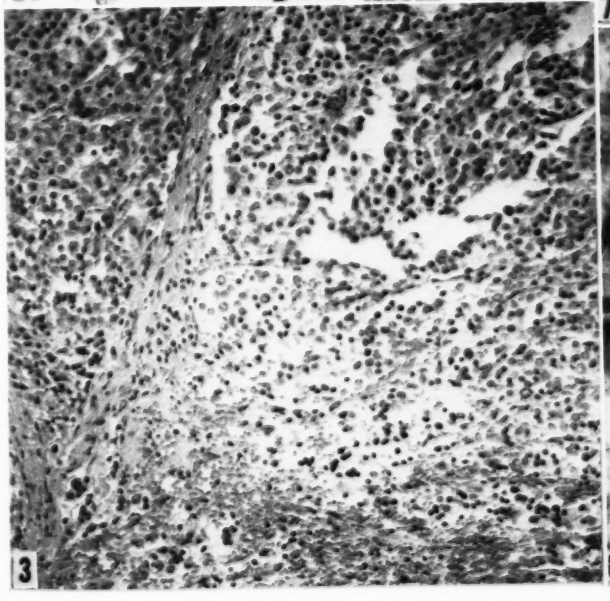
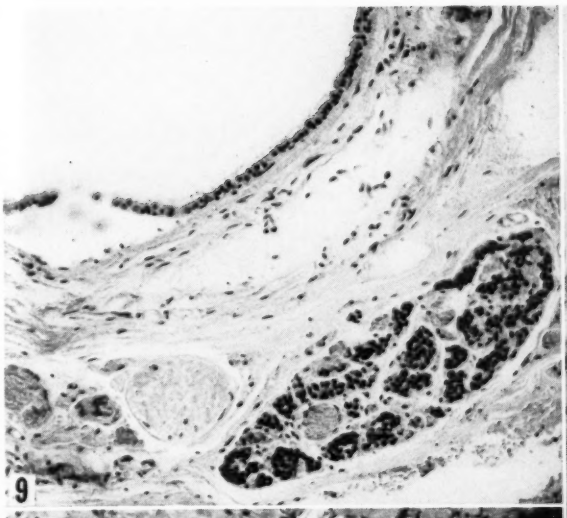


FIG. 9.—The site of the thyroid in a mouse that had been given 269 μc. of I^{131} 14 months before death is indicated by the parathyroid, which is undamaged, a stenosed artery ("radiation arteritis"), and a nerve bundle. ×75.

FIG. 10.—The site of the thyroid in a mouse that had been given 375 μc. of I^{131} $3\frac{1}{2}$ months before death indicated by thick-walled stenosed arteries, chronic diffuse and perivascular inflammation, fibrosis, and degeneration with ossification of the adjacent part of tracheal cartilage. ×100.

FIG. 11.—Residual thyroid tissue with fibrosis of the surrounding area in a mouse that had been given 75 μc. This animal had the smallest thyroid of mice so treated, and the grafted pituitary tumor grew to be large. ×240.

FIGS. 12 and 13.—Small thyroid of a mouse that had been given 75 μc. Note the fibrosis, moderate anaplasia, and intracytoplasmic colloids. The grafted tumor (Strain 3D) measured 10 mm. in average diameter. Fig. 12, ×240; Fig. 13, ×970.



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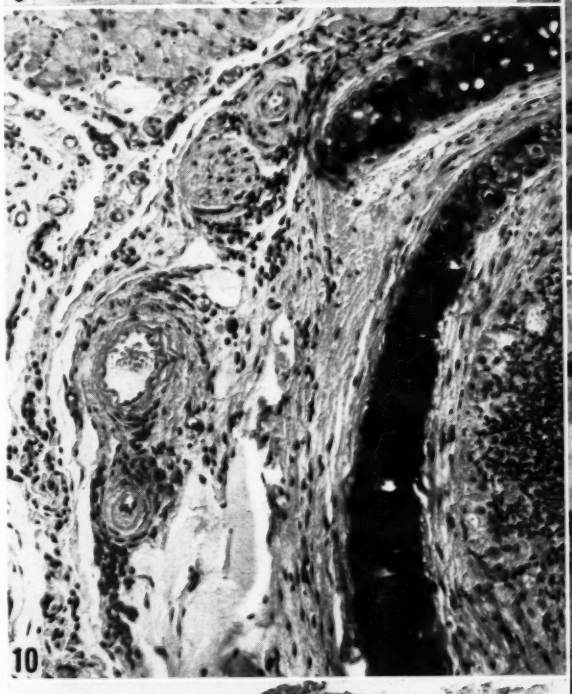

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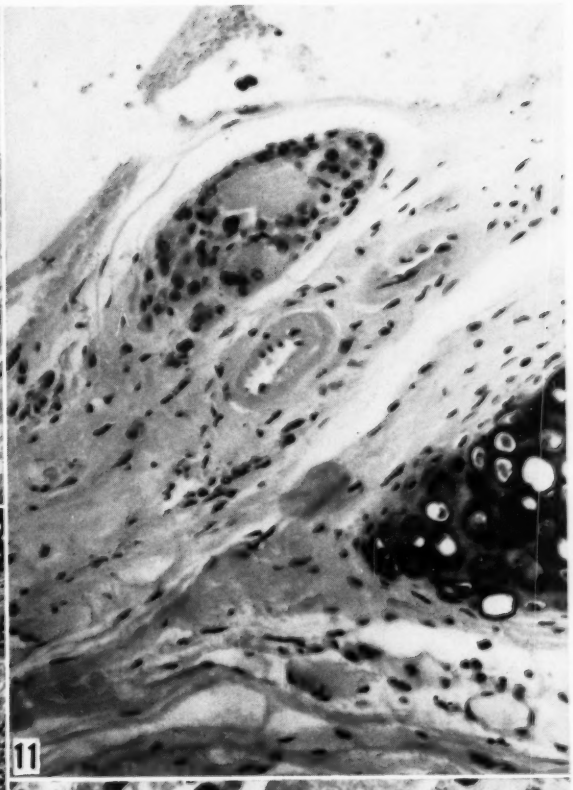

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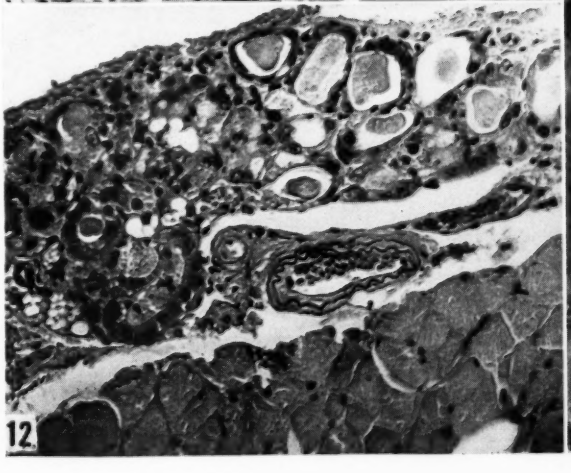

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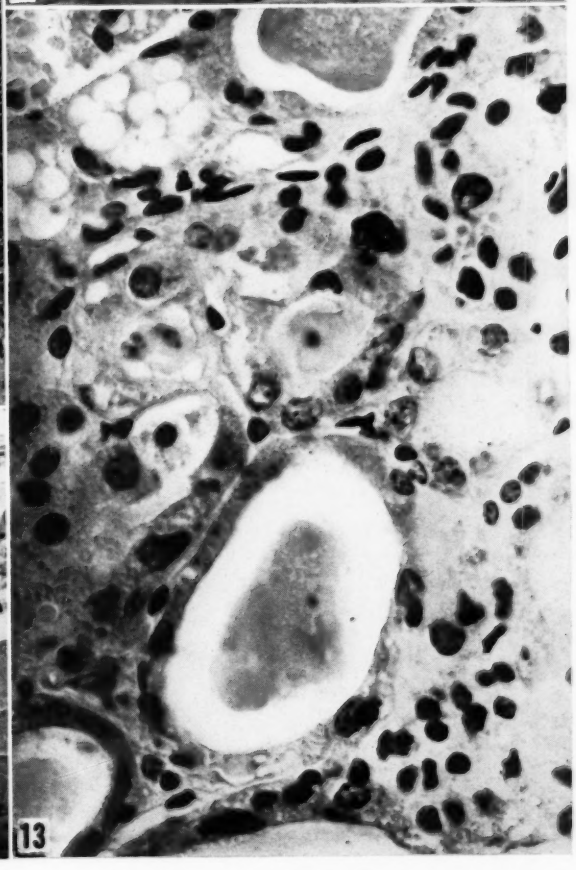

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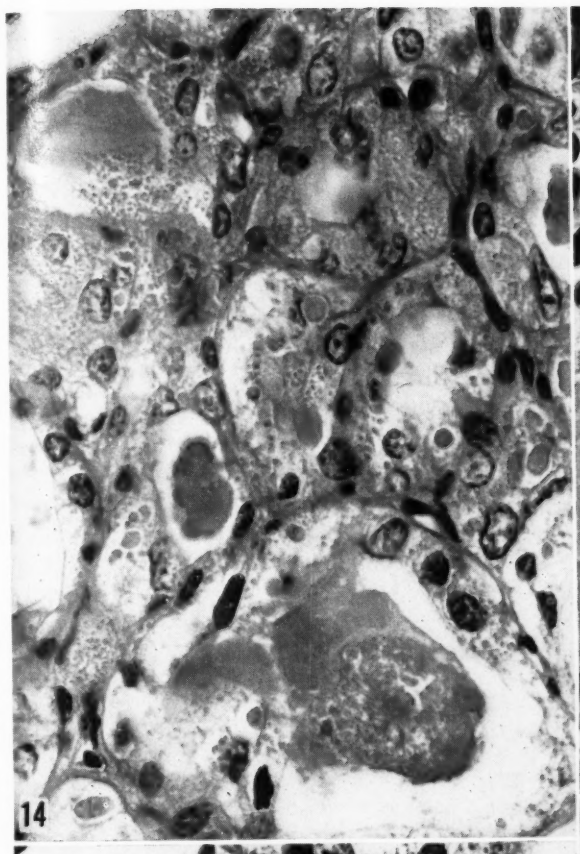
FIG. 14.—The thyroid of a mouse stimulated with TSH of the grafted pituitary tumor and injured but slightly by an antecedent injection of 75 μc. Note the swelling of the cells, anaplasia, variability in quantity and staining properties of the colloid in the acini and intracytoplasmic colloid globules. $\times$970.

FIG. 15.—The thyroid of a mouse 7 months after administration of 25 μc. of I^{131} not grafted with tumor. The organ was approximately normal in size, but the microscopic appearance is indicative of slight stimulation. $\times$970.

FIG. 16.—Highly stimulated thyroid of a mouse given 25 μc. followed by a dependent pituitary tumor graft which measured 1.5 cm. in average diameter. $\times$970.

FIGS. 17 and 18.—Autoradiographs of the thyroid of a mouse given 25 μc. and bearing a grafted dependent pituitary tumor (Strain 3) of 2 cm. in average diameter. The thyroid was greatly enlarged and had numerous adenomas. The latter failed to take up I^{131} which accumulated in the normal appearing acini as indicated by the black granules of reduced silver. $\times$100.



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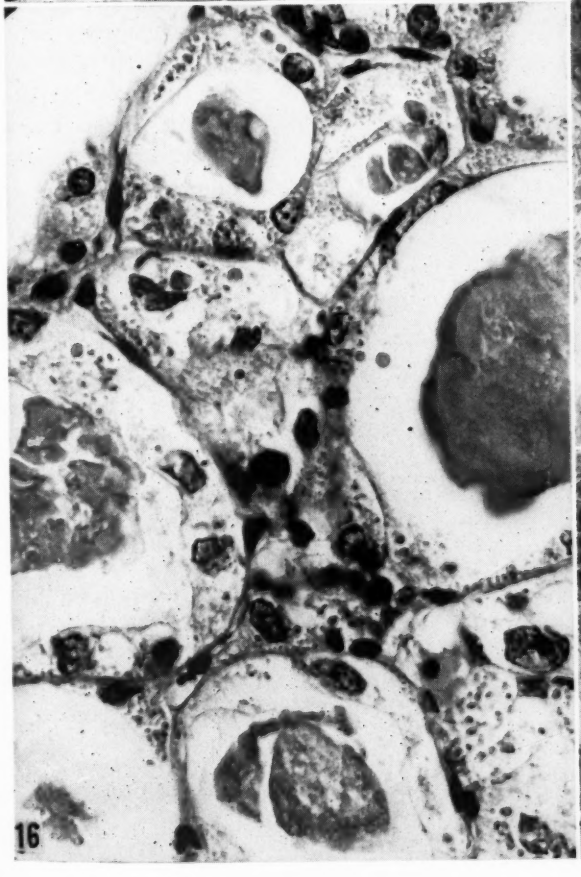

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dine. Little, if any, of the I^{131} retained in thyroidectomized mice is expected to be in thyroid hormone; even if extrathyroidal TH synthesis occurs, the total amount of hormone produced is evidently below that required to compensate for the absence of the thyroid.

SUMMARY AND CONCLUSIONS

The relationship of growth of TSH-secreting pituitary tumors to thyroid function was studied by destroying or depressing the thyroid gland of mice with graded doses of I^{131}, grafting tumors on such animals, and correlating tumor growth with thyroid function. The results indicate that proliferation of grafted TSH-secreting pituitary tumor cells depends on the secretion of TH.

Near complete or complete destruction of the thyroid follows administration of a single dose of 200 or more μc. of I^{131}. This provides the maximum stimulus to pituitary growth. A lower dose, about 75 μc., produces a lasting depression of the thyroid, some follicles of which, although normal in appearance, are incapable of undergoing fully compensating hyperplasia. In most mice 25 μc. interferes little, if at all, with the thyroid function, with ability of this gland to be stimulated by TSH, and with its capacity to inhibit the growth of pituitary tumors. The inverse relationship between thyroid function, as indicated by its ability to take up I^{131}, and stimulation of the pituitary tumor grafts is almost quantitative.

Cells of thyroid glands which escaped destruction by large doses of I^{131} do not have the ability to undergo a fully compensating hyperplasia. This may be due in part to the stenosing "radiation arteritis" and diffuse fibrosis in the thyroid region caused by I^{131}.

Radiothyroidectomy causes an increased total-body retention (or diminished elimination) of I^{131} during the first 24 hours following injection. Pituitary tumor grafts secreting TSH further enhance total-body retention of I^{131}. The sites of this I^{131} are not precisely known. In radiothyroidectomized mice (with and without tumor) the elimination of the retained I^{131} is rapid. In normal mice bearing grafted tumors most of the retained radioiodine is in the thyroid, and its retention is lasting.

ACKNOWLEDGMENTS

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18. SEIFTER, J.; EHRICH, W. E.; and HUDYMA, G. M. Effect of Prolonged Administration of Antithyroid Compounds on the Thyroid and Other Endocrine Organs of the Rat. Arch. Path., **48**:536–47, 1949.
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Announcements

Meeting of the Radiation Research Society

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

Erratum

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 821; substitute for the last paragraph:

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O_2 consumption, it is seen that the amount of glucose "cleavage products" *exceeds* the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and *is exceeded* by the glucose utilized by 16 per cent in CLL. If the assumption is made that, *in this respect*, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if $M = +0.27$ and $L = -0.16$ and the normal differential is 65 per cent M and 35 per cent L, then

$$0.65\ (+0.27) + 0.35\ (-0.16) = +0.12\ ,$$

a figure identical to the observed +0.12 for normal leukocytes.